THE IMMUNOBIOLOGY OF *HELIGMOSOMOIDES POLYGYRUS* IN THE MURINE HOST

by

Catherine Elizabeth Lawrence, B.Sc (Hons)

Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy, October, 1990.

CONTENTS

ABSTRACT

The development of the gastrointestinal nematode *Heligmosomoides polygyrus* (syn. *Nematospiroides dubius*) in the mouse was studied. The stage specific production of acetylcholinesterase was measured in both excretory/secretory products and in worm homogenates and found to be maximal between days 4-6 post infection, corresponding to the fourth larval stage of the parasite's life cycle.

Analysis of the proteolytic enzymes found in the same preparations of the parasite again revealed a stage specific release. Quantitative examination showed a maximum concentration of proteolytic enzymes in the early third larval stage, whilst qualitative analysis revealed a number of molecules at 96, 76, 42, 33, 18, 16, and 13 kDa in the early stages, which gradually disappeared as the parasite aged until only those at 76, 18, 16, 13 kDa remained by day 120.

The molecules present on the surface of the various stages of the parasite were extracted using a number of procedures. Various stage specific surface molecules were identified as were two possible sex specific molecules at 76 and 145 kDa.

The immune response to a primary infection of the parasite was characterised in three strains of mice with different degrees of susceptibility to infection (SJL, BALB/c and CBA). It was noted that the better the strain was at expelling the parasite, the greater and swifter was the response as assessed through the use of a number of criteria. These included white blood cell counts, differential cell counts, the B and T cellularity of the secondary lymphoid organs, the response of these cells to mitogens, the mucosal mast cell response, quantitative antibody response (Mancini and ELISA) and qualitative antibody response to parasite antigens (immunoblot). In each case SJL responded better than BALB/c which, in turn responded to higher degree than CBA.

Functional host protective immunity was stimulated in the same three strains of mice using a challenge infection following a 9-day anthelmintic abbreviated infection. The same criteria were used to measure the immune response to the parasite as for the primary infection and, as for the primary infection, it was found that the high responder strains gave a more rapid and more intense reaction to the parasite than the low responder strain. Immunisation prevented the establishment of a proportion of the challenge infection and also resulted in the premature expulsion of parasites.

The parasite surface molecules which were recognised by mice undergoing either a primary infection or an immunising infection were identified. It was revealed that molecules at 208, 145, 92, 76 and 62 kDa on adult parasites were recognised by mice which had expelled a primary infection. Mice which were immune to a challenge infection recognised molecules at 62 and 20-15 kDa on larval parasites. A molecule at 30.5 kDa was also recognised by immune mice and corresponded to the molecular weight of acetylcholinesterase in the ES.

DEDICATION

To Mum, Jerry and Paul

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank my supervisor Dr. David Pritchard for his invaluable advice, enthusiasm and encouragement.

Particular thanks go to Drs. J.M. Behnke, P.G. McKean and R.K. Grencis and Mr. K.V. Legget for their generous help and co-operation with much of the work in this thesis and to Professors D. Wakelin and P.N.R Usherwood for providing the necessary facilities and a conducive atmosphere. Thanks also go to Pfizer and Dr. Ken Gration for the support of the CASE studentship.

Finally, to all of my friends and colleagues, Drs. Brailsford, Bolas-Fernandez, Crook, Else, Lammas, Mellor, Rogan, Tighe and Wells and Linda, Maura, Richard, Liz, Dave, Doreen, Nigel and Faisal and especially Lisa, thanks for a very enjoyable three years. The contribution of the technical staff, Jill, Sue, Keith and Hugh is much appreciated as is that of Anthony Pitt for his photography. I also wish to thank Prof. O. Jarrett at Glasgow Universisty for the provision of the word processing facilities and those in the Feline Virus Unit for their encouragement.

ABBREVIATIONS

A	ampere
AChE	acetylcholinesterase
APS	ammonium persulphate
B-ME (2-ME)	beta mercapto-ethanol
CO_2	carbon dioxide
Con A	concanavalin A
Coomassie	Coomassie Brilliant Blue
cpm	counts per minute
CTAB	cetyltrimethylammonium bromide
DTH	delayed type hypersensitivity
ELISA	enzyme linked immunosorbent assay
EPG	eggs per gramme faeces
ES	excretory/secretory products
FCA	Freund's complete adjuvant
FCS	foetal calf serum
FDA	fluorescein diacetate
FIA	Freund's incomplete adjuvant
g	gramme (weight), gravity
HRP	horse radish peroxidase
Hom.	parasite homogenate
Ig	immunoglobulin
IL-1,2,3,4	interleukin-1,2,3,4
kDa, kD	kilodaltons
KLH	keyhole limpet haemocyanin
l	litre
L1, L2, L3, L4	first, second, third, fourth larval stage
LPS	lipopolysaccharide
M	molar
MLN	mesenteric lymph nodes
MMC	mucosal mast cell
MW	molecular weight
NMS	normal mouse serum
NRS	normal rabbit serum
NS	not significant

OD	optical density
O/N	overnight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PChE	pseudocholinesterase
p.i.	post infection
RMCP II	rat mast cell protease type II
S1	PBS extracted cuticle
S2	SDS extracted cuticle
S3	B-ME extracted cuticle
SDS	sodium dodecyl sulphate
TBS	tris buffered saline
TCA	trichloroacetic acid
TEMED	tetraethylmethylene diamine
Tris	Tris (hydroxymethyl) amino methane
V	volt
VCU	villus crypt unit(s)
WBC	white blood cells

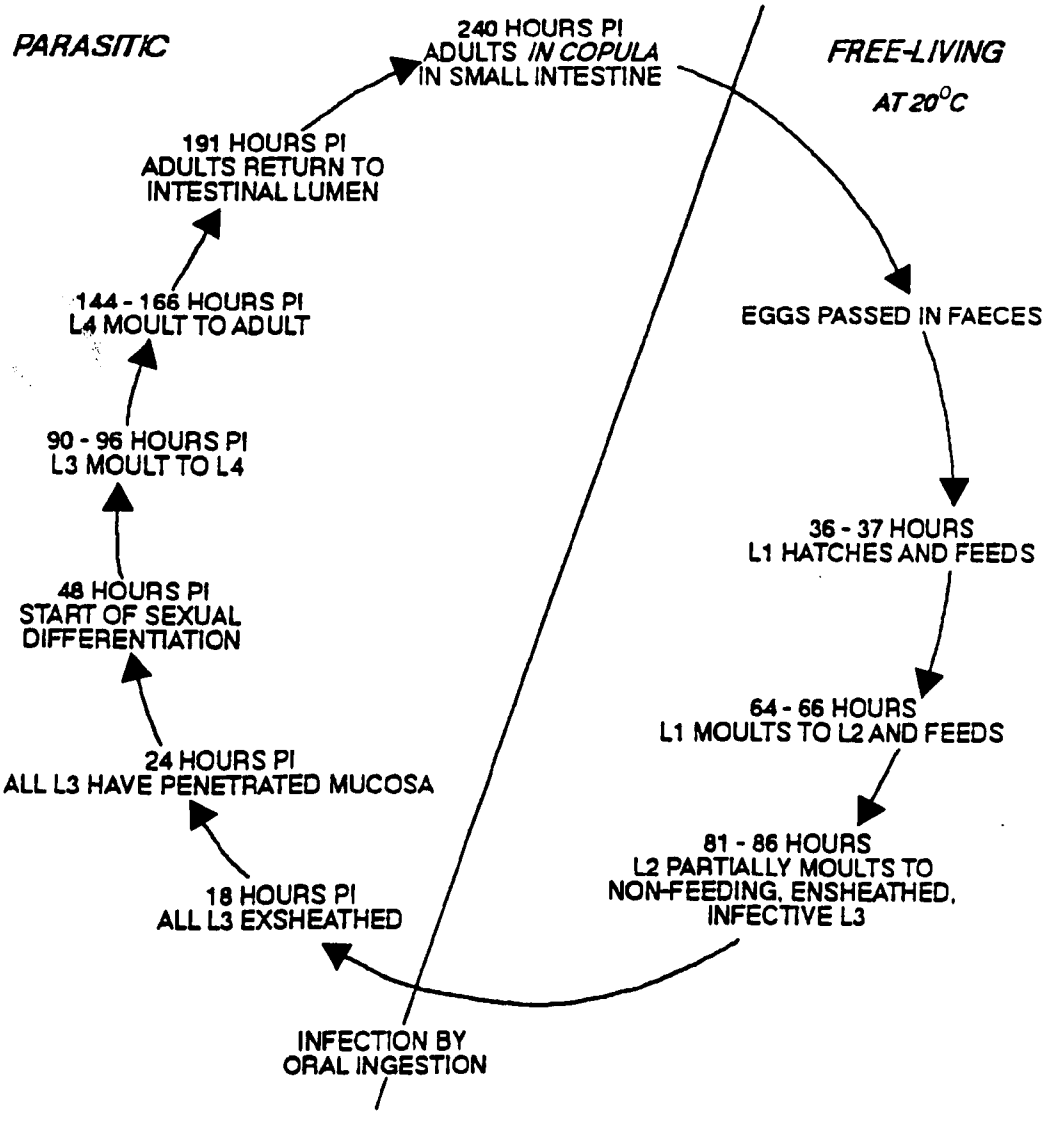
CHAPTER ONE
GENERAL INTRODUCTION

GENERAL INTRODUCTION

Heligmosomoides polygyrus (syn. *Nematospiroides dubius*, Baylis, 1926) is a gastrointestinal trichostrongyle nematode parasite of mice. The life cycle is simple and direct, involving both free-living and parasitic stages. The most detailed examination of the life cycle of this parasite was done by Bryant (1973) and revealed a number of features (Fig. 1.1). After the eggs are laid they hatch and release a first stage rhabditiform larva (L1); this feeds on bacteria in the faeces and then moults to give rise to the second larval stage (L2), after a further period of feeding the parasite undergoes a partial moult to the third stage filariform larva (L3), which is active but non-feeding. The time taken for this series of events to occur is primarily dependent on temperature, the optimum being 20°C, but humidity, oxygen tension and the population of bacteria present are also important. Infection occurs by the oral ingestion of the ensheathed third stage larvae. The sheath is the partially shed cuticle of the second larval stage and it serves to protect the worm against the harmful effects of microbial attack, pH in the host stomach and proteolytic host enzymes. However, the sheath is slightly permeable, allowing gases such as carbon dioxide and oxygen and ions to enter in order to allow both the respiration and detection of stimuli to occur so that the parasite can exsheath in the host. The main stimuli for exsheathment appear to be temperature, pH and pCO_2 (Sommerville and Bailey, 1973). After exsheathment, the parasite invades the muscularis mucosa of the anterior small intestine where it undergoes two further moults from the third larval stage (L3) to the fourth larval stage (L4) on day 4 post infection (p.i.) and from an L4 to the adult on day 6-7 p.i. Both of these moults occur whilst the parasite larvae are encysted in a host granuloma in the mucosa. By day 8 the adult parasites begin to migrate to the intestinal lumen where they start feeding, mating and egg production, the first eggs being produced by day 10 p.i.

Figure 1.1 Life Cycle of *Heligmosomoides polygyrus*.

The life cycle of *H. polygyrus* was constructed using data from Bryant (1973). The times for the development of the free-living stages are given in hours post hatching at $20^{o}C$. The times for the parasitic stages are given in hours post infection (p.i.).


PARASITIC
240 HOURS PI
ADULTS IN COPULA
IN SMALL INTESTINE
FREE-LIVING
AT 20°C
191 HOURS PI
ADULTS RETURN TO
INTESTINAL LUMEN
144 - 166 HOURS PI
L4 MOULT TO ADULT
EGGS PASSED IN FAECES
90 - 96 HOURS PI
L3 MOULT TO L4
36 - 37 HOURS
L1 HATCHES AND FEEDS
48 HOURS PI
START OF SEXUAL
DIFFERENTIATION
64 - 66 HOURS
L1 MOULTS TO L2 AND FEEDS
24 HOURS PI
ALL L3 HAVE PENETRATED MUCOSA
81 - 86 HOURS
L2 PARTIALLY MOULTS TO
NON-FEEDING, ENSHEATHED,
INFECTIVE L3
18 HOURS PI
ALL L3 EXSHEATHED
INFECTION BY
ORAL INGESTION

Once the parasites are in the lumen they not only aggregate within the intestine but they also aggregate with respect to each other (Lewis and Bryant, 1976). The anterior aggregation of the parasites close to the mucosa in the duodenum was proposed to be due to a number of factors including increased oxygen tension, decreased pH, supply of dietary nutrients and the presence of bile salts (Panter, 1969a). The aggregation of parasites with respect to each other was attributed to sexual chemoattraction, the more parasites that are in a particular space the more likely it will be that mating can occur. Thigmokinesis was also proposed as a reason for this aggregation; Lee (1969) suggested that these responses would be advantageous in helping to maintain the position of the parasite between the villi on the intestinal mucosa. Alternatively, it was implied that a local increase in the concentration of parasite products could have a greater effect on the host and so possibly delay its expulsion. These parasite products could include enzymes such as acetylcholinesterase, which is thought to act as a `biological holdfast', or proteases, which could enable the parasite to feed, or immunomodulatory factors, which are thought to suppress the host immune response to the parasite. Therefore any one, or a combination of, these factors could result in the delayed expulsion of the parasite and thus increase the time available for reproduction.

Although the parasite feeds on host gut contents and not on blood and tissues like the human hookworms *Necator americanus* and *Ancylostoma duodenale*, it is capable of inducing extensive pathology in the host intestine (Liu, 1965a, b). The pathogenesis of this nematode parasite has been compared, in certain respects, with that induced by the trichostrongylids of domestic animals (Baker, 1954). The majority of the pathology caused is due to the penetration of the host mucosa by the larvae. However, it has been proposed that the larvae release substances which are able to lyse the surrounding tissue and destroy the glandular epithelium (Liu, 1965a; Baker, 1954). The lesions induced in reinfected mice are much greater than

those seen in a primary infection (Liu, 1965b) and it was suggested that this was due to hypersensitivity to larval products.

H. polygyrus is extensively used as a murine model of chronic gastrointestinal nematode infection; it is also used, but not to the same extent, as a model for the study gastrointestinal trichostrongyle parasites of veterinary importance and medical importance, such as *Haemonchus contortus*, *Trichostrongylus colubriformis* and endemic human hookworm. *H. polygyrus* is a long lived parasite and can survive as a primary infection, in naive hosts, between 8-10 months (Robinson, Wahid, Behnke and Gilbert, 1989). This system is a better model for the study of chronic gastrointestinal nematodes than that of the rat strongylid, *Nippostrongylus brasiliensis*. Although *N. brasiliensis* is commonly used to study the immunological events accompanying the expulsion of nematode parasites, it does not provide a useful model for the study of chronic nematode infections as this parasite is expelled from a primary infection in naive animals between 12 and 15 days p.i. The *H. polygyrus*/mouse model also has its advantages over the *N. americanus*/hamster and the *A. ceylanicum*/hamster models. Not only does the *N.americanus*/hamster model not allow the study of challenge infections as infection of the animals has to occur when they are neonates, but the number of immunological reagents directed against hamster molecules is severely limited. The number of mouse reagents, on the other hand, is plentiful, allowing not only the contribution of various antibody classes to be assessed but the role of the various cell types and cytokines can be analysed. The study of the genetic control of the immune response to the parasite can also be assessed through the use of inbred strains of mice. This is pertinent to the study of human infection as it has been demonstrated that there are groups of people who have high worm burdens which cannot be accounted for by exposure alone and vice versa and so it has been proposed that there is a genetic control of immunity to gastrointestinal nematodes (Wakelin, 1986). As there are few

inbred hamster strains and hamsters are monomorphic at MHC I, there is a limit to the degree to which the genetic control of immunity to infection can be investigated in this model system. Therefore, a much more detailed analysis of the factors inducing a chronic infection can be performed using the *H. polygyrus*/mouse system. However, there are disadvantages in using this model as the parasite molecules which would be present in a human and/or veterinary infection cannot be studied and thus the role of protective antigens, through the use of infected human and animal sera, cannot be assessed.

The use of malnourished mice infected with the parasite (Slater and Keymer, 1986; Brailsford and Mapes, 1987) showed that protein deficiency increased the effects of the parasite upon the host. The cell mediated immunity appeared to be depressed and this was accompanied by a synergistic hypoalbuminaemia. Similar interactions are thought to occur in humans infected with gastrointestinal nematodes and thus, the use of this host/parasite system seems to provide an excellent model for the study of the interactions between the host immune system and the parasite evasion strategies.

Infection with *H. polygyrus* significantly impairs the expulsion of other parasites such as *Trichinella spiralis*, *N. brasiliensis*, *Hymenolepis diminuta* and *Trichuris muris* (Behnke, Wakelin and Wilson, 1978; Hopkins, 1980; Jenkins and Behnke, 1977; Colwell and Wescott, 1973), whereas infection with *T. spiralis* concurrently with *H. diminuta*, *H. microstoma* or *T. muris* (Behnke and Wakelin, 1977; Howard et al, 1978; Bruce and Wakelin, 1977) results in their premature loss when *T. spiralis* is rejected. One of the proposed mechanisms for the survival of a chronic infection of this parasite in the mouse host is that of immunodepression of the host by the parasite which not only suppresses the response to the parasite but it also suppresses the response to heterologous challenge. Decreased antibody titers have been reported in mice concurrently infected with influenza virus (Chowaniec, Wescott and Congdon, 1972) and a general suppression of circulating antibody to sheep erythrocytes was reported in infected mice (Shimp,

Crandall and Crandall, 1975; Ali and Behnke, 1983; Pritchard, Ali and Behnke, 1984). A depression of DTH responses of mice infected with this parasite has also been reported (Price and Turner, 1986a,b, 1987).

The ability of a wide range of both protozoan and helminth parasites to cause immunosuppression has been extensively studied (Playfair, 1982; Ogilvie and Wilson, 1976; Behnke, 1987). A decreased inflammatory response may actually be advantageous to the host, since it is an abnormal immune reaction which usually induces the pathology in the first place. For instance, immune responses to eggs in schistosome infections, microfilariae in *Onchocerca volvolus* and adult parasites in *Wuchereria bancrofti* infections cause the resulting pathology. It appears that the host and the parasite need to strike a fine balance. The host needs to respond to the parasite in order to eliminate it, but an increased response to it could induce severe pathology. The parasite needs to immunomodulate the host to such a degree that it facilitates its survival but not to such an extent which would result in the death of the host due to infection with other opportunistic infections.

It appears that parasites employ numerous mechanisms for the modulation of the immune response. Parasite molecules are thought to activate host cells which regulate immune responses, such as thymus-dependent (T) suppressor lymphocytes and adherent suppressor cells (Ottesen, Weller and Heck, 1977; Piessens et al, 1982; Weller, 1978; Lammie and Katz, 1983). The suppression of granulomatous hypersensitivity in *Schistosoma mansoni* infections (Coulis, Lewart and Fitch, 1978) can be transferred with cells from the spleen and lymph node cells but not with the serum from chronically infected mice. The depression of several thymus dependent responses occurs when egg production begins. Jirds infected with *Brugia pahangi* (Leiva and Lammi, 1989) develop suppressive mechanisms after an initial phase of vigorous immunologic responses to parasite antigens during the prepatent period. The appearance of microfilariae in the blood is associated with the induction of

immunoregulatory circuits in the spleen. An initial response followed by suppression is also noted in *Trypanosoma cruzi* infections (Grossman, Greenblatt and Cohen, 1986). Infections elicit a T helper response which can be observed 4-6 days p.i., concomittant with this is the induction of suppressor cells capable of reducing the response to heterologous antigens or haptens. However, these cells cannot regulate the parasite induced T helper cells until 12-14 days p.i. When this occurs the regulation is sudden and profound, depressing the response by up to 90%. This period of immunostimulation followed by immunosuppression is analogous to the series of responses to *H. polygyrus* in mice. The larval stages of this parasite appear to be immunogenic, whilst the adult stages are immunomodulatory, suppressing the response to both heterologous and homologous stimuli (Ali and Behnke, 1983; 1984; Behnke, Ali and Pritchard, 1983; Cayzer and Dobson, 1983; Colwell and Wescott, 1973; Crawford, Behnke and Pritchard, 1989; Dehlawi and Wakelin, 1988; Jenkins and Behnke, 1977;Losson, Lloyd and Soulsby, 1985; Monroy, Dobson and Adams, 1989; Price and Turner, 1983a,b,c; 1987; Pritchard, Ali and Behnke, 1984; Shimp, Crandall and Crandall, 1975).

Other proposed mechanisms for the generation of immunosuppression in a wide variety of parasitic diseases have included antigenic competition, non-specific T cell suppression, blocking effects of soluble antigens or antigen-antibody complexes, specific suppressor T cells, suppressor adherent cells (macrophages), serum suppressive factors (cytokines) and T cell tolerance to parasite antigens (Raybourne, Desowitz, Kliks and Deardoff, 1983; Leiva and Lammie, 1989). The enhancement of number and/or function of suppressor T cells has been demonstrated in a number of parasitic infections including, *T. cruzi* (Liew, Scott, Liu and Croft, 1987), *H. polygyrus* (Pritchard, Ali and Behnke, 1984; Price and Turner, 1986a,b), *Ascaris suum* (Soares, Macedo and Mota, 1987) and *Oesophogastomum radiatum* (Gasbarre, Romanowski and Douvres, 1985).

In many cases it appears that immunosuppression is limited or concentrated to the spleen. For instance, in jirds infected with *B. pahangi* (Leiva and Lammie, 1989) the immunosuppressive phenomena are not observed in the lymph nodes and are apparently anatomically restricted to the spleen. Indeed, there is some evidence that suggests that the spleen is relatively rich in suppressor cells compared to the peripheral blood (Rich and Rich, 1974). Depletion of suppressor cells from the spleens of *B. pahangi* infected jirds (Leiva and Lammie, 1989) restores the proliferative responses to parasite antigens. The responses of the MLN cells were equal to those from controls but the proliferation and production of IL-2 of spleen cells was suppressed. Therefore, it is evident that the parasite could be activating the immune regulatory mechanisms and it was suggested that non-specific suppressor cells were responsible rather than an inherent defect in the ability of macrophages to stimulate the cells.

The generation of T suppressor cells has been implicated in infection with *T. cruzi*, as depletion of the Lyt 2^+ subset results in the restoration of the immune response to the infection (Grossman, Greenblatt and Cohen, 1986). The exact sequence of events which lead to the generation of these suppressor cells and the ultimate modulation of the host immune response has not been fully elucidated but it appears that the following processes may occur. The parasite releases some substance which may mediate directly or indirectly through a second messenger, which then either suppresses macrophage activation or induces suppressor macrophages and/or suppressor T cells. This then results in a general or specific suppression of the host immune response depending on the exact mode of action of the parasite substance.

It has been demonstrated (Bielefeldt-Ohmann, Filion and Babiukl, 1983) that although macrophages are required for the induction of suppressor cells they may also be suppressive themselves. For instance, the macrophage is thought to be the cell primarily involved in the generation of a defective antibody response to *T. cruzi* (Reed, Pihl and Grabstein, 1989)

as the addition of IL-1 and IL-2 to infected mice restores the B cell response. IL-1 stimulates T helper cell function, thus causing the expansion of B cell clones and so the restoration of the response with external IL-1 indicates that the helper T cells are functional but are not being activated by macrophages. In the case of toxoplasmosis (Luft, Pedrotti and Remington, 1988) adherent suppressor cells are implicated in the specific suppression of the response to the parasite. In infection with *S. mansoni*, a macrophage-like suppressor cell causes the depression of several thymus dependent responses to soluble egg antigen (Coulis, Lewart and Fitch, 1978) and in *Ostergia ostertagi* infections, the presence of low serum antibody levels, despite repeated exposure, is thought to be due to macrophages not producing sufficient IL-1 to drive blastogenesis (Cross and Klesius, 1989).

IL-2 also appears to be involved in the immunosuppression of antibody responses (Beltz and Kierszenbaum, 1987). It was shown that immunosuppression of *T. cruzi* infected hosts induced the alteration of accessory cell function, reduced levels of T cells in the spleen and altered lymphocytes producing antibody. Despite low levels of IL-2, responsiveness could not be restored with exogenous IL-2. IL-2 was also shown to be responsible for the general lymphocyte unresponsiveness in *Plasmodium falciparum* infections (Ho and Webster, 1989). Lysis of T suppressor cells, had no effect on the response and there were no adherent suppressor cells present, however, there was a defect in the IL-2 production and/or receptor expression as addition of exogenous IL-2 did not reconstitute the response. Therefore it was suggested that this was the result of irreversible cell alteration or the reduced capacity of the cell to bind and/or respond to IL-2 and/or the need for additional cytokines.

Prostaglandins have been associated with the generation of immunosuppression. In syphillitic rabbits, the suppressive component was present in the macrophage population. Addition of PGE_2 increased the suppression whilst the addition of IL-1 and IL-2 partially restored the response. *Treponema pallidium*

produces PGE_2 which causes macrophages to produce more PGE_2, which in turn, causes the suppression of proliferation (Tomai, Elmquist, Warmka and Fitzgerald, 1989). Prostaglandins have also been implicated in *Trichostrongylus colubriformis* infections (Fransden and Bone, 1989). Other inflammatory mediators such as mast cell released histamine (Dohlsen, Sjogren and Carlsson, 1987) are also thought to contribute to indirect suppression of the hosts immune response. These are thought to induce suppressor macrophages which act on other cells (Regan, Cohen, Cromartie and Schwab, 1988). These suppressor macrophages are thought to be initially induced by the cell walls of normal gut flora bacteria as sterile animals showed no such suppressor cells (Mattingley, Eardley, Kemp and Gershon, 1979).

Interferon is also suspected as being involved in the control of suppression. Gamma-interferon was shown to activate macrophages, but during toxoplasmosis the levels were reduced (Diez, Galdeano, Nicolas and Cisterna, 1989). In contrast, the levels of alpha- and beta- interferon were elevated and these were thought to inhibit lymphocyte proliferation and antibody synthesis.

The exact nature of the parasite-derived molecules responsible for the immunosuppression of the host have yet to be elucidated but it has been shown that excretory/secretory (ES) products of *Leishmania tropica* (St. Charles, Franf and Tanner, 1980), larval *T. spiralis* (Faubert, 1976), adult *S. mansoni* (Camus, Dessaint, Fischer and Capron, 1977), *H. polygyrus* (Pritchard, Ali and Behnke, 1984; Reed, Dehlawi and Wakelin, 1988; Losson Lloyd and Soulsby, 1985; Monroy and Dobson, 1989; Pritchard, Appleby and Lawrence, unpublished data), *N. americanus* and *A. ceylanicum* adults (Garside, McKean, Behnke and Pritchard, 1989) have immunosuppressive properties. A heat labile substance of greater than 10 kDa from *Anisakis simplex* suppresses mitogen-induced blastogenesis (Raybourne, Desowitz, Kliks and Deardoff, 1983). Adult schistosomes produce a 500-1000 Da heat stable product which was thought to be a prostaglandin. This inhibits the mitogen-induced

proliferation and is found both in the culture supernatants and serum of infected rats (Dessaint, Camus, Fischer and Capron, 1987). A high molecular weight product from *O. ostertagi* larvae was capable of modifying the humoral and cellular response of the host (Cross and Klesius, 1989) and *B. malayi* microfilariae contain and release molecules of high molecular weight that suppress the mitogen-induced *in vitro* proliferation of lymphocytes (Wadee, Vickery and Piessens, 1987).

Leid, Suquet, Bouwer and Hinrichs (1986) demonstrated that *Taenia taeniaeformis* produces a proteinase inhibitor, taeniastatin, which causes the suppression of proliferation. It was proposed that this blocks the generation of IL-2 and inhibits the action of IL-1 and this was due to the inhibition of the proteinase which is required for the cleavage of IL-2 for its activation. The saliva of the tick *Ixodes damini* also inhibits IL-2 production and it was proposed that this was either due to the presence of a proteinase inhibitor or a prostaglandin.

T. cruzi suppressor substance (Liew, 1988) was shown to be a substance of 14-15 kDa which suppresses the response of spleen cells to mitogens and unrelated antigens. It was thought to be associated with T helper cells and appeared to be activated or cleaved by some other factor. It was suggested that this was again due to the presence of a proteinase inhibitor which acts on IL-2 in a way which causes the malfunction and non-specific inhibition of proliferation.

Parasite proteins with direct suppressor activity are distinct from parasite mitogens that might indirectly contribute to immune suppression via the polyclonal activation of the hosts immune system. This could explain the apparent paradox of polyclonal hypergammaglobulinaemia in infections where there are no specific antibodies to parasite antigens (Wadee, Vickery and Piessens, 1987). Phosphorylcholine has been shown to be present in large amounts in nematode antigen fractions (Maizels, Burke and Denham, 1987) and it has been shown that it may be capable of inducing B cell tolerance (Mitchell and Lewers, 1976).

From these studies it would appear that immunosuppression of the host could be due to a defective and/or suppressed Th_1 subset as many of the suppressive activities exerted by parasites are manifested by a decreased and/or defective production of IL-2 and gamma-interferon. Thus, it would seem that the parasite could quite ably manipulate the cellular response to itself without affecting the antibody response as the Th_2 subset appears unaffected. In fact, in the case of *H. polygyrus* infection there is a potent antibody response to the parasite but there appears to be a defect in the cellular response.

A causal relationship between parasite burden and immune impairment has been demonstrated (Feldmeir et al, 1988). In a schistosome infection the numbers of helper/inducer cells drop and the numbers of suppressor cells rise and this appears to be related to the parasite burden. Elimination of the parasites with drugs leads to a normalisation of the immune response similar to that seen with the cure of leprosy and leishmaniasis (Carvalho, Teixeira and Johnson, 1981; Mshana, Haregewoin and Belehu, 1982). Thus, it appears that modulation of the immune response is associated with active infection and may be reversible. This may also help to explain why large infections with *H. polygyrus* result in chronic infections in some strains whilst low intensity infections are expelled (Wahid, Robinson and Behnke, 1989).

The ability of *H. polygyrus* to depress homologous immunity has been amply demonstrated. It has been shown (Cayzer and Dobson, 1983) that infections removed with anthelmintic give better protection than an infection superimposed on an existing parasite population. The extended survival of the parasites in concurrently reinfected mice was related to reduced anti-*H. polygyrus* antibody levels. Behnke, Hannah and Pritchard (1983) demonstrated that although a very strong immunity is elicited by irradiated larvae, if normal larvae are administered at the same time, the immunity is depressed, but if the adult parasites are removed by anthelmintic treatment, the resistance to challenge is

restored. It was also demonstrated that mice immunised with irradiated larvae could be immunodepressed by transplanting adult parasites into the mouse intestine (Behnke, Williams, Hannah and Pritchard, 1987).

The proposed methods of immunomodulation of the host by *H. polygyrus* have included, reticuloendothelial blockade, prevention of antigen processing in the major lymph organs, enzymatic degradation of antigen, induction of immunological tolerance and the induction of suppressor macrophages and/or T cells. The mechanism of immunosuppression with most evidence to support it is the generation of non-specific suppressor cells. The presence of suppressor T cells was initially demonstrated by Pritchard, Ali and Behnke (1983). Spleen cells from animals injected with adult antigen were capable of transferring suppression and it was shown that the cells responsible were T suppressor cells. Price and Turner (1983a,b; 1986b; 1987) have also illustrated the presence of T suppressor cells and suggested that they were specific for DTH responses but non-specific for antigen.

The generation of immunosuppressive macrophages was suggested to be responsible for the observed lesion in both the afferent and efferent arms of the immune response (Price and Turner, 1987) and that this was mediated by prostaglandins (Price and Holt, 1986) which were able to suppress the proliferative responses of lymphocytes to both mitogen and parasite antigens. Crawford, Behnke and Pritchard (1989) demonstrated that suppressor macrophages could be induced by both larval and adult parasites, although that by the adult was greater. They proposed that the polyanionic molecules known to be present on the nematode cuticle were associated with the activation of these macrophages.

The fact that there is a substantial hypergammaglobulinaemia concurrent with an increased IgG_1 catabolism (Brown, Crandall and Crandall, 1976; Pritchard, Williams, Behnke and Lee, 1983) has been proposed as an alternative mechanism for immunosuppression. Increased IgG_1 catabolism following a response to parasite antigens could mean that any

protective responses generated against the parasite would be quickly metabolised and thus depress any protective response. It has also been proposed that elevated IgG_1 titers could block potentially host-protective responses and that IgG_1 immune complexes could activate suppressor T cells (Mitchell, Goding and Rickard, 1977; Mitchell et al, 1977). However, it was later shown that no blockage was evident (Pritchard, Williams, Behnke and Lee, 1983).

Overall, it appears that there may be two mechanisms for the generation of immunosuppression by this parasite. One is thought to be antigen specific and may be caused by the induction of suppressor macrophages and/or T cells. The factors released by the activation of these cells then leads to the generalised suppression of both the hosts humoral and cellular responses. It was suggested (Price and Turner, 1983c) that the activation of hepatic or alveolar macrophages by systemic contact with parasite material provided an explanation for the enhancement of `T helper independent' antibody responses by intestinal parasites, whilst persistent circulating antigen was responsible for the generation of T suppressor cells. It has been demonstrated that both suppressor macrophages and T cells are active in this infection (Pritchard, Ali and Behnke, 1984; Price and Turner, 1983a,b,c; 1986b; 1987) and that there is a defective IL-2 production in a chronic primary infection (Prowse, Mitchell, Ey and Jenkin, 1979). Cells from the spleens of infected animals are unable to support mast cell growth (Reed, Dehlawi and Wakelin, 1988). Reed *et al* (1988) suggested that this was either due to the presence of suppressor cells or a defect in cytokine production. The systemic depression of DTH and inflammatory responses is likely to have considerable implications on gut immunity. Indeed, it is systemic suppression that retards the rejection of other gastrointestinal nematodes in concurrent infections, as *T. muris* which occupies the large intestine, well away from any local effects that *H. polygyrus* may have induced, has a delayed expulsion when mice are concurrently infected with *H. polygyrus*.

A number of attempts have been made to isolate the parasite molecule/s responsible for the immunosuppression. Monroy, Dobson and Adams (1989) identified a molecule in adult ES that was able to inhibit both mitogen and antigen induced proliferation. The factor was less than 26 kDa and not inactivated by SDS or boiling. Losson, Lloyd and Soulsby (1985) identified molecules of less than 14 kDa from larval parasites and in the serum of infected mice which suppressed the lymphoproliferative response to mitogens. Price and Turner (1983a) demonstrated the presence of an immunomodulatory factor which was inactivated by heating and trypsin. It has also been shown that a factor present in adult ES at 55 kDa was capable of suppressing the antibody response of spleen cells to keyhole limpet haemocyanin (KLH) *in vitro* (Pritchard, Appleby and Lawrence, unpublished data). Although extracts of this parasite have been shown to contain proteolytic enzymes (Cayzer and Dobson, 1984; Monroy, Cayzer, Adams and Dobson, 1989) these were thought to be unlikely to be the immunosuppressive factor, both due to the fact that serum contains a number of protease inhibitors (Ohlsson and Tegner, 1973).

Although immunodepression of the host by parasites has been widely demonstrated in a range of parasitic diseases, the relevance to parasite survival is not certain. Parasite species such as *N. brasiliensis* (Price and Turner, 1986a) are rapidly expelled from the host. However, there are cases where there is still an antibody response to the parasite despite a parasite-induced immunodepression (Macaskill, Holmes, Jennings and Urquhart, 1981). There is a substantial antibody response against *H. polygyrus* but it is not expelled from a primary infection suggesting that although the afferent arms of the immune system may be functional, there is a lesion in the efferent arm, limiting the inflammatory and cellular responses to the parasite and thus delaying its expulsion. Despite the obvious parasite induced immunomodulation and its implications for the survival of the parasite in the host, an immune response can be generated against the parasite and a number of effective immunisation protocols have been devised. These

usually involve the prolonged exposure of the host immune system to larval antigens, either through the use of irradiated larvae, multiple infections, anthelmintic abbreviated infections or the ectopic injection of larvae or their products (Behnke and Robinson, 1985; Behnke and Wakelin, 1977; Enriquez, Cypess and Wassom, 1988; Ey, 1988a,b; Goven and De Buysscher, 1980; Hagan, Behnke and Parish, 1981; Hosier, Sackman and Idell, 1974; Jones and Rubin, 1974; Mitchell and Munoz, 1983; van Zandt, 1973). Although some adult antigens have also been used, they usually require purification and it is proposed that this is due to the presence of immunomodulatory factors in crude preparations.

The aim of this work was to make a full dissection of the host/parasite relationship in the *H. polygyrus/* mouse system. In order to do this it was decided to approach the task in two ways, firstly, the changes in functional enzymes, notably AChE and protease enzymes, and structural cuticular proteins would be monitored to see if this could be linked in any way to the observed immunostimulation by the larval stages and the immunomodulation by the adult stages. Secondly, the immune response to the parasite would be studied in three strains of mice, SJL, a high responder, BALB/c, an intermediate responder and CBA, a low responder. The response to both a chronic, primary infection and to an immunising anthelmintic abbreviated infection would be monitored in order to clarify which portion of the immune response i.e. humoral or cellular, is responsible either for the rejection of a primary infection and/or immunity to challenge. The study of a number immunological parameters including peripheral WBC counts, differential cell counts, the cellularity of the secondary lymphoid organs and the numbers of T and B cells in them, the response of these cells to mitogen, the mucosal mast cell response, the antibody response to parasite antigen and the total antibody concentration will all be measured and correlated both to the responsiveness of the host and the immunity to the parasite.

The first approach allows for the determination of the possible immunogenic molecules in larval stages and the

relevance of stage specific cuticular proteins, proteases and AChE production to the immunogenicity of the larval stages. This may also enable the elimination of molecules which could be immunomodulatory and so should allow the molecule responsible for this to be identified more readily. The isolation of stage specific molecules, both cuticular proteins and enzymes would also help in identifying why a response to the larval antigens has such a damaging effect whilst an antibody response to the adult parasite appears to have little effect on it survival.

The second approach allows for the detailed dissection of the immunological events accompanying the expulsion of a primary infection of the parasite from various strains of the mouse host. The factors that make some mouse strains more or less able to reject the parasite, i.e. the speed of generation of the immune response, the strength of the response or maybe the ability to recognise some unique molecule, the destruction of which results in the expulsion of the parasite, can also be investigated. The study of the immune response to challenge following an immunising anthelmintic abbreviated infection allows us to determine which portion of the immune response causes immunity to challenge and why prolonged exposure of the immune system to larval antigens is able to do this. Larval antigens may stimulate some part of the immune response which adult antigens suppress or they may promote the recognition of antigens unique to the larval stage whose recognition results in the impaired development and/or destruction of the stage before it matures, whilst any surviving adult worms are unaffected.

In summary, the aim of this study is to examine various aspects of the host/parasite relationship and to try to correlate changes in the parasite with vulnerability to expulsion and the immune response of various strains of the mouse host with the ability to reject the parasite. It is hoped that this multi-faceted approach will answer some of the questions about the survival of a chronic nematode infection in the face of a potentially damaging immune response.

CHAPTER TWO

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Animals

Outbred male or female CFLP mice were obtained from the breeding colony maintained at the Nottingham University Zoology Department animal house. These mice were used for the passage of the parasite and for the collection of the various stages of the parasite. Syngeneic female SJL, BALB/c, CBA and C57BL/10 were bought in from Harlan-Olac Ltd, Oxon. Animals were used at 6-8 weeks of age, and on receipt were given two doses, one week apart of piperazine citrate via a gavage needle in order to remove any pinworm before infection. Animals were kept under standard animal house conditions and pelleted food (Pilsbury's, Ltd.) and tap water were available *ad libitum*.

2.2 Parasite

The strain of *Heligmosomoides polygyrus* (syn. *Nematospiroides dubius*) used was obtained from the Wellcome Research Laboratories, Beckenham, Kent in 1975 and maintained as a stock infection in outbred CFLP mice.

2.2.1 Parasite Maintenance

Mice harbouring patent infections (> 20 days p.i.) were placed in wire bottomed cages which were suspended over trays containing wet tissue paper. After 24 hours, the mice were replaced in their original cages and the faecal samples collected in a beaker. The faeces were then mixed with a small amount of powdered charcoal and distilled water until a smooth paste was produced. This mixture was then thinly spread onto damp 9 cm filter papers which were placed in glass petri dishes and the lids put on. The dishes were then placed in a large plastic box on top of damp tissue paper. This was then placed in an incubator at 24-26^{o}C for 6-8 days, by which time the infective third stage larvae had accumulated around the edge of

the filter paper and in the bottom of the petri dish. The filter papers were removed and gently washed with distilled water into the petri dish and then the contents of the dish were poured into a glass conical flask. The larvae were allowed to settle overnight at $4^{o}C$, washed twice with distilled water and adjusted to the appropriate concentration before use. Larvae older than 21 days were discarded.

2.3 Parasitological Techniques

2.3.1 Infection of Animals

Animals were inoculated directly into the stomach lumen via a blunt ended 21 G needle with an appropriate number of infective larvae suspended in no more than 0.25 ml.

2.3.2 Recovery of Third and Fourth Larval Stages

Infected animals were killed by inhalation of chloroform and larvae were recovered using a modified Baermann technique. The entire small intestine was removed and opened longitudinally. The mucosa was removed by scraping it off with a microscope slide and the intestine and the debris accumulated on the slide, placed on a square of nylon gauze which was then suspended in a 50 ml beaker containing Hanks' saline. The beaker was then incubated at $37^{o}C$ in a water bath for 3-4 hours. After this time, the gut and gauze was removed and the larvae sedimented and washed several times in warm Hanks' saline followed by PBS and were then ready for use.

2.3.3 Recovery of Adult Worms

Animals were killed as above and the entire small intestine removed, slit longitudinally and placed on a square of nylon gauze. This was then placed in a 50 ml beaker containing Hanks' saline and incubated at $37^{o}C$ for 3-4 hours. If required for antigen or culture they were washed as for the

third (L3) and fourth (L3) larval stages, however, if they were being counted, after 3 hours incubation, the temperature of the water bath was increased by $1^{o}C$ every 15 mins. for 1 hour. This causes the parasites to `untangle' and thus be easily counted under a dissecting microscope.

2.3.4 Measurement of Parasites

Parasites were preserved in formalin/ethanol (1:1 v/v) until required. They were then drawn with the aid of a camera lucida attached to the dissecting microscope and the drawings were measured with the aid of a bit-pad digitiser linked to an Apple PC.

2.3.5 Faecal Egg Counts

Faecal egg counts were carried out by the standard zinc sulphate flotation method (Behnke and Parish, 1979b). One gram of pooled faeces (dry) deposited over a 25 hour period was taken from each experimental group. 10 ml of 50% saturated sodium chloride solution were added to each sample and left for 3-4 hours at room temperature to allow dispersal of the faecal pellets. The contents were thoroughly, but gently, mixed with the aid of a flea and a magnetic stirrer. 50 ml of 40% zinc sulphate solution (see Appendix) were added and the mixture poured through a sieve. After flotation in a standard McMaster counting slide, the eggs were counted under a microscope and the quantity expressed as number of eggs per gram of faeces (EPG).

2.3.6 Artificial Exsheathment of L3

It is possible to affect exsheathment of infective larvae (L3) by treatment with bleach. However, the effects that this may have on the larvae is not known and so a more controlled method was employed. Briefly, the technique used was that described by Cypess, Pratt and van Zandt (1973). Larvae were

suspended in 0.01 M sodium tetraborate, gassed with 40% CO_2, 60% N_2 for 10 mins. and incubated for 30 mins. in a shaking water bath at 37°C. Up to 75% of the larvae were observed to be totally exsheathed. The larvae were then separated from the cast sheaths by sedimentation.

2.4 Parasite Antigen Preparation

2.4.1 Phosphate Buffered Saline Homogenate

Adult, L4 and L3 parasites were recovered using the various methods previously described. They were then homogenised, on ice, in a minimum volume of PBS with a ground glass tissue homogeniser. When the parasites were completely disintegrated, the insoluble material was removed by centrifugation at 10,000g for 10 minutes and the resulting supernatant removed, aliquoted and stored at -40°C until required.

2.4.2 Excretory/Secretory (ES) Products

The procedure for the collection of parasite excretory/secretory (ES) products was essentially the same as that described by Carr and Pritchard (1986). Adult, L4 and L3 parasites were collected as previously described and washed extensively in Hanks' saline. Dead or damaged worms were removed and the rest were placed in a sterile petri dish containing 1% penicillin and 1% streptomycin. After three washes in this medium, the parasites were then incubated at 37°C for 24 hours, after which the supernatant was collected, this process was continued for 96 hours after which time the parasites started to die. The supernatants were filter-sterilised in order to remove worm debris and eggs and then dialysed against distilled water for 24 hours at 4°C to remove the phenol red dye present in the medium The dialysate was frozen at -80°C, lyophilised, diluted to a suitable concentration, aliquoted and stored at -40°C until required.

2.4.3 Parasite Culture on Collagen Rafts

Collagen rafts were prepared in 3cm sterile petri dishes by initially spreading 0.95ml of stock collagen solution (1g rat tail collagen fibres in 125ml of sterile 0.1% acetic acid) evenly onto each dish (Wilde, Hasan and Mayer, 1984). 0.2ml of a mixture of RPMI 1640 and sterile 0.3N NaOH (2:1) was added with mixing, and the gels set at 37°C. Before use the rafts were equilibrated with RPMI 1640 overnight at 37°C. Parasites were then cultured for ES products as above.

2.5 Radio-labelling Techniques

2.5.1 ^{125}I Radio-labelling of Parasite Surfaces

Iodination of proteins containing a tyrosine residue involving the oxidation of $Na^{125}I$ and the subsequent incorporation of radio-iodine into the tyrosine molecule is one of the commonest methods employed. Some workers have reported that iodine may also react with other amino acids such as histidine (Covelli and Wolff, 1966), tryptophan (Koshland, Engelberger and Gadone, 1963) or sulphydryl groups (Jovanovic, Nemoda and Lwin, 1972), but tyrosine is the principal amino acid involved. Some incorporation of radio-active iodine can be found in neutral and polar lipids (Watt, Burgess and Metcalf, 1979).

Labelling with ^{125}iodine was performed using the water insoluble chloroamide, 1,2,3,4,6-tetrachloro 3a, 6a diphenyl-glycouril (Iodo-gen, Pierce Chemical Co.) as described by Fraker and Speck (1978).

Iodo-gen was dissolved in methylene chloride to give a concentration of 1 mg/ml and 50 ul of this solution was then added to 0.75 x 10 cm glass tubes and left to evaporate overnight in a fume hood at room temperature. The coated tubes were then wrapped in foil and stored in a desiccator at 4°C until required.

The tubes were rinsed with PBS before use in order to remove any flakes of Iodo-gen reagent. 100 ul of packed worms were placed in the bottom of the tubes and the volume made up to 200 ul using PBS. Care was taken to ensure that no damaged parasites were used and so only truly surface proteins were labelled.

The reaction was initiated by the addition of 10 ul of 11 ug/ml potassium iodide (KI) in PBS, followed by 1 MBq of ^{125}I. Although this is not a large amount of radio-iodine, good incorporation was achieved, the parasites were still alive and the proteins retained their immunogenicity. The tubes were agitated gently for 10 mins. to ensure that all of the parasites had been labelled and then the reaction was terminated by washing the worms from the tubes with excess PBS. Extensive washing with large volumes of PBS was continued until the supernatant was free of radio-activity.

2.5.2 Iodine Labelling of Proteins

100 mg of protein in 100 ul of PBS was added per tube followed by 10 ul of 11 ug/ml KI solution and 2 MBq of ^{125}I. After 10 mins. the reaction was stopped and the unreacted iodine removed by gel filtration. This was done using a pre-packed Sephadex G 25 column (Pharmacia). The labelled protein was added and washed through with PBS, the 2 ml void volume was collected and discarded and the following 5 mls were collected in 1 ml fractions. These fractions were analysed for labelled protein by TCA precipitation and those with the highest percentage of TCA precipitable counts were retained and stored at $4^{o}C$ for use within 1 week.

2.5.3 Cuticle Solubilisation Procedure

The method used was essentially that of Pritchard, McKean and Rogan (1988) adapted from Cox, Kusch and Edgar (1981). Labelled parasites were homogenised in PBS and centrifuged at 10,000g for 5 mins. and the resulting supernatant designated

S1. The pellet was washed with fresh PBS and centrifuged as before, thus ensuring that proteins did not contaminate the next preparation. The pellet was then treated with an SDS buffer (see Appendix) for 3 mins at 100°C and then centrifuged, this supernatant was designated S2. The pellet was washed twice in SDS buffer and then treated with SDS buffer containing 5% B-ME for 3 mins. at 100°C. Centrifugation of this gave a supernatant, S3 and a B-ME insoluble pellet.

2.5.4 CTAB Stripping of Parasites

The proteins of the nematode cuticle can be selectively removed using the cationic detergent cetyltrimethylammonium bromide (CTAB) (Pritchard, Crawford, Duce and Behnke, 1985). Stripping the parasites in 0.25% CTAB w/v in Eagles MEM for 4 hours at 37°C was found to remove the majority of the labelled protein from the surface of the parasite without causing any gross damage to the worm. Parasites were incubated in CTAB either before or after the labelling procedure. The supernatant from the unlabelled parasites was discarded whilst that from the labelled worms was collected, centrifuged at 10,000g for 10 mins. to remove any debris and then dialysed overnight at 4°C in order to remove both the detergent and any free iodine. The supernatant was then assessed for TCA precipitable counts before use.

2.5.5 Triton X 114 Extraction of Hydrophobic Proteins

Triton X 114 is a non-ionic detergent and homogeneous at 0°C, but separates into an aqueous and detergent phase above 20°C. This allows phase separation of hydrophilic and hydrophobic proteins from parasite surfaces (Bordier, 1981).

Labelled parasites were washed extensively in PBS and then homogenised on ice in Triton X 114 buffer (see Appendix). After centrifugation at 10,000g for 10 minutes in a chilled centrifuge at 4°C, the supernatant was removed. This was carefully layered onto a sucrose cushion (see Appendix) and

incubated at 30°C for 3 mins. Following centrifugation at RT for 5 mins. the upper aqueous phase was removed. Triton X 114 was then added to this supernatant in sufficient quantities to make a 1% solution (v/v). The supernatant was then carefully added to the initial sucrose cushion and the procedure repeated as before. Finally, the upper aqueous phase was removed, followed by the sucrose plug and then the detergent phase, taking care not to contaminate any of the preparations. Each sample was then made up to the same buffer and detergent concentrations. The amounts of TCA precipitable material was assessed and the samples stored at 4°C for use within 1 week.

2.5.6 Trichloroacetic Acid Precipitation of Labelled Protein

Trichloroacetic acid (TCA) precipitations of the labelled material was used to determine the amount of labelled protein in each sample.

10 ul of labelled samples were added to 100 ul of 25% TCA and 30 ul of FCS and then incubated on ice for 20 mins. The solution was centrifuged at 10,000g for 5 mins. and the resulting supernatant removed. Both the supernatants and the pellets were counted on a Packard Auto Gamma 800c counting system. The counts per minute (cpm) of the pellet and the supernatant were used to calculate the percentage of labelled protein present in the sample, thus;

$$\% \text{ TCA precipitable} = \frac{\text{cpm in pellet}}{\text{cpm in pellet + supernatant}} \times 100$$

2.5.7 Microplate Assay for the Quantification of Protease Enzymes

This was a simple sensitive assay, which made use of a substrate, casein, which can be digested by a broad spectrum of enzymes. This assay was essentially that described by Robertson, Kwan-Lim and Maizels (1988) but using casein as the substrate rather than gelatin.

Briefly, 500 mg of casein was labelled and purified as described in 2.5.2. The labelled protein was counted and then diluted in 0.06 M carbonate buffer pH 9.6 (see Appendix) to give 600,000 TCA precipitable cpm/ml. This was then used to coat a microtitre plate with 10,000 cpm/well and incubated overnight at $4^{o}C$. After extensive washing, 3 x 10 mins PBS, 2 hours PBS at $37^{o}C$ and finally 3 x 10 mins. with PBS, the number of counts that had bound to the plate were assessed using a gamma counter as before. Finally, the extracts to be tested were added, 10 ug of protein (ES or homogenate) were added in 50 ul of PBS pH 8.0 in triplicate. A standard trypsin curve was also set up using a range of trypsin concentrations between 10 ug/well to 0.001 ug/well in the same volume and buffer as the test samples. The plate was then incubated at $37^{o}C$ for 4 hours and at the end of this time the supernatants were removed. Both the supernatants and the wells were counted on the gamma counter and the percentage of labelled substrate released by the samples was calculated as follows;

$$\% \text{ substrate released} = \frac{\text{cpm in supernatant}}{\text{cpm in supernatant + cpm in well}} \times 100$$

The equivalent tryptic activity was calculated by subtracting the background value (% substrate released in the presence of PBS only) and then using the trypsin standard curve. The value obtained was expressed in tryptic units; mg tryptic activity per mg sample protein. Therefore, the activities of the various samples could be compared.

2.6 Biochemical Techniques

2.6.1 5-20% Gradient SDS-Polyacrylamide Gel Electrophoresis

The method used was essentially that of Laemmli (1970). See Appendix for preparation of solutions and buffers. Glass plates were cleaned with 70% ethanol and assembled according to

the instructions in the LKB manual. 16 ml of the 20% acrylamide solution was poured into the mixing chamber with the outlet tap, and 16 ml of the 5% solution was added to the other chamber. Next, 10 ul of TEMED (N,N,N,N-tetramethyl-ethyldiamine, Sigma), an accelerator of polymerisation, and 70 ul of 10% ammonium persulphate (APS) the initiator of polymerisation were added to each chamber. The outlet tap and the tap between the two chambers were immediately opened and the solutions allowed to pour gently between the plates. A magnetic stirrer in the outlet chamber ensured that the 20% solution was well mixed with the 5% solution. Once the chambers were emptied, methyl-propan-1-ol was carefully used to overlay the gel and produce a smooth, even upper surface. After about 1 hour when the gel had set, the methyl-propan-1-ol was thoroughly washed off with distilled water and the stacking gel poured on the top of the resolving gel with the appropriate well-former in place. Once this gel had polymerised, the combs were removed and the wells washed with running buffer (see Appendix). Samples were then loaded in a maximum of 100 ul in sample buffer (see Appendix) and the gels run overnight at $10^{o}C$ (LKB 2219 Multitemp Unit) at a constant voltage of 70 V on a LKB13 2001 vertical electrophoresis unit powered by a LKB 2197 transformer. The next morning the voltage was increased to 400 V and when the dye from had reached the bottom, the gels were taken off. Gels were removed from the plates and the stacking gel cut off. They were then ready for staining or Western blotting.

2.6.2 Sample Preparation

Protein samples (100 mg/lane 10 wells; 1 mg/lane single well) were added to an equal volume of sample buffer and boiled at $100^{o}C$ for 5 mins. Samples were loaded on the gel using an Ultipette micropipettor.

2.6.3 Gel Staining

Gels were stained for protein using Coomassie Brilliant Blue (see Appendix). They were placed in the stain overnight on a rocking platform, followed by 2-3 days in 2 changes of destain (see Appendix) until the background was minimal and protein bands were visible. The gels were then photographed and dried down onto 3 MM filter paper on a Bio-Rad 443 Slab Dryer at 60^{o}C for 4 hours.

2.6.4 Substrate Gels

The incorporation of proteolytic substrates into SDS-PAGE gels at the time of polymerisation does not alter the mobility of the polypeptides during electrophoresis and can serve as substrates for the detection of enzymes in the picogram range (Spanos and Hubscher, 1983). This technique combines the advantages of enzyme identification with the resolution of SDS-PAGE (Lacks and Springhorn, 1980).

Casein (`Carnation' evaporated milk) was used as the substrate. This was incorporated into 12% resolving gels at a final concentration of 1% (see Appendix). 100 ug of non-reduced samples of PBS-extracted homogenates or parasite ES were run overnight at 4^{o}C, 70 V constant voltage. The next morning, the gels were washed in 3 changes of 2.5% Triton X 100 (v/v) in distilled water for 1 hour in order to remove all the SDS and so allow re-naturation of the enzymes, followed by 30 mins. in 2 changes in distilled water.

Digestion was allowed to take place by incubating the gels for 24-48 hours in 0.1 M glycine NaOH pH 8.0 at 37^{o}C. The gels were stained and destained with Coomassie Brilliant Blue as before, until clear areas of enzymic activity were visible against a blue background.

2.6.5 Protein Estimation

The method used for the estimation of protein concentration was a modification of that described by Lowry, Rosebrough and Farr (1951). Dilutions of a standard concentration protein solution (bovine serum albumin, BSA, 1 mg/ml) and the test samples were made up in distilled water to a volume of 250 ul. 1.25 ml of working reagent (see Appendix) were added to each tube and incubated at 37^{o}C for 5 mins. Next, 125 ul of a 1:1 dilution of Folin and Ciocalteu's phenol reagent:water were added, mixed and the tubes incubated at 37^{o}C for 15 mins. Duplicates of 100 ul of each sample were transferred to the wells of a microtitre plate and the optical density (OD) read at 630 nm on a Dynatech MR700 plate reader. A calibration curve of OD versus standard protein concentrations was drawn and the concentration of the test samples calculated.

2.6.6 Autoradiography

Stained and dried down gels were carefully stuck inside a film cassette with intensifying screens. The gels were then exposed to Kodak X Omat film at -80^{o}C for varying periods of time dependent on the amount of label loaded onto the gels. The films were developed and fixed in Agfa Gevaert G150 and G350 fixer and developer according to the manufacturers instructions. The molecular weight standards were marked on the film and the film photographed.

2.6.7 Acetylcholinesterase Assay

The method employed for the measurement of acetylcholinesterase activity in samples of both parasite ES and homogenates was essentially that described by Ellman, Courtney, Andrew and Featherstone (1961). 10 ul of sample were diluted to 3 ml in phosphate buffer pH 8.0 (see Appendix). To each sample and an enzyme free blank 20 ul of substrate were

added (see Appendix). Acetylthiocholine (ATC) was used to measure the acetylcholinesterase levels and butrylthiocholine (BTC) was used to measure the pseudocholinesterase levels. Next, 100 ul of reagent (dithiobisnitrobenzoic acid, DTNB (see Appendix)) was added to each tube and immediately 2 X 100 ul aliquots were taken, put in a microtitre plate and the optical density read at 410 nm on a Dynatech MR700 plate reader (T = 0). Following incubation of the tubes at 37^{0}C further readings were taken at 5, 15, 30, 45 and 60 mins. The rate of enzyme activity in mol/l/min was calculated using the following equation;

$$\text{Rate} = \frac{\text{change in absorbance/min}}{1.36 \times 10^{4}}$$

The specific activity (mol/l/min/mg of parasite protein) of each sample was then calculated.

2.7 Immunochemical Techniques

2.7.1 Western Blotting

The objective of the Western blotting technique is to demonstrate the recognition of individual components from a complex antigenic mixture by antibodies. After proteins had been separated by gradient SDS-PAGE they were transferred onto nitrocellulose essentially according to Towbin, Stahelin and Bordon (1979). The gel was briefly washed in blotting buffer (see Appendix) and carefully placed onto a buffer soaked sheet of 3 MM filter paper. A sheet of nitrocellulose was cut to size, soaked in blot buffer and gently placed on top of the gel ensuring that there were no air bubbles trapped between the two. A further layer of soaked filter paper was placed on top of the nitrocellulose and then all five layers were sandwiched between soaked blotting sponges and gratings from the LKB blotting apparatus. The unit was then fitted into the blotting

tank with the nitrocellulose facing towards the anode. It was then subjected to electrophoresis for 90 mins. at 10°C at a constant current of 1 A. After transfer, the nitrocellulose was dried and the marker lane cut off. If pre-stained markers had not been used, the lane was stained for 2 mins. with Amido black (see Appendix) and destained until the protein bands were visible.

2.7.2 Probing Western Blots with Mouse Anti-sera

The nitrocellulose from the blotted samples was cut into 5 mm strips with a scalpel and the remaining protein binding sites on the nitrocellulose were blocked by incubating in blocking buffer (see Appendix) for 1 hour at room temperature on a rocking platform. The strips were then incubated overnight at 4°C on a rocking platform in 4 ml of a solution of the test serum at a 1 in 250 dilution in blocking buffer. The following day, the strips were removed from these solutions and washed 3 x 10 mins in TBS-Tween (see Appendix). The strips were then incubated for 2 hours at room temperature on a rocking platform in a 1:1000 solution of peroxidase conjugated sheep anti-mouse IgGAM (Serotec) diluted in TBS-Tween. The strips were then washed again, as before, in TBS-Tween, followed by 5 mins. in distilled water and then incubated in peroxidase substrate (see Appendix) until optimally developed (10-30 mins.). Finally, the blot was rinsed and placed on used X-ray film and photographed whilst still wet.

2.7.3 Probing Western Blots with Rabbit Anti-sera

The same protocol was used as for mouse anti-sera but the rabbit anti-serum was added at a dilution of 1:500. The conjugate used was peroxidase conjugated Protein A at a dilution of 1:1000.

2.7.4 Immunoprecipitation of Labelled Material

Up to 50 ul of radio-labelled proteins (10-50,000 cpm) were added to 25 ul of test sera and made up to 150 ul with immunoprecipitation buffer (see Appendix). This was then incubated overnight at 4°C. The next morning, 20 ul of sheep anti-mouse IgGAM (Serotec) was added to each tube and incubated for 2 hours at room temperature. The precipitates were then centrifuged at 10,000g and the resulting pellet washed 4 times with ice-cold immunoprecipitation buffer. Finally, the pellets were boiled in 25 ul of reducing sample buffer for 5 mins. before being resolved by SDS-PAGE as above. The gels were stained, dried down and then subjected to autoradiography.

2.7.5 Enzyme Linked Immunosorbent Assay

Serum antibody responses to parasite ES antigens (adult and day 4) were determined by a standard enzyme linked immunosorbent assay (ELISA). A solution of ES protein was prepared at the desired concentration (5 ug/ml) in 0.05 M carbonate/bicarbonate buffer pH 9.6 (see Appendix) and 50 ul aliquots were added to each well of a 96 well flexible microtitre plate (Falcon) which was then incubated overnight at 4°C. The next morning the plate was washed 5 times in wash buffer (PBS, 0.05% Tween 20) with the last two washes including a 5 minute incubation in the wash buffer. The plate was then blocked against non-specific binding by incubation with 100 ul of 10% solution of skimmed milk (Marvel) in wash buffer for one hour at room temperature. Plates were then washed twice in wash buffer and 50 ul of test sera of the appropriate dilution (1:100 in wash buffer) was added to the wells in triplicate and incubated at room temperature for 90 mins. The plates were washed a further 5 times as before and then 50 ul of an alkaline phosphatase-conjugated goat anti-mouse IgGAM (Sigma) at a dilution of 1:350 in wash buffer was added. Following incubation at ambient temperature for 90 mins. the plate was washed for a final 5 times and 100 ul of standard substrate

(see Appendix) was added to each well. The plate was wrapped in aluminium foil, to exclude light and prevent evaporation, and incubated at room temperature for 30 mins. After this time the optical density (OD) of the wells was read at 410 nm on a Dynatech MR700 plate reader.

2.7.6 Single Radial Immunodiffusion (Mancini)

The measurement of the concentration of mouse immunoglobulin classes in serum was done using the Mancini technique (Mancini, Carbonara and Hereman, 1965) with IgG, IgA and IgM radial immunodiffusion kits (The Binding Site, Ltd.). each kit consisted of one plate for the measurement of IgG, IgA and IgM. The plates were set up according to the manufacturer's instructions. Briefly, one standard for each plate was made up and loaded in 5 ul in one well. Next, each test sample was diluted appropriately and added to the plate in 5 ul. The plates were incubated at room temperature until judged complete when the standard ring diameter had achieved a certain size. In this case this was 24 hours for IgG, 48 hours for IgA and 72 hours for IgM. Once the test rings were clearly visible they were measured with the aid of an immunodiffusion reader and the concentrations of each sample calculated using the tables provided.

2.8 Cellular Techniques

2.8.1 Collection of Blood

Mice were bled fortnightly from the tail. The animals were restrained in a pierced 50 ml syringe with the end removed. The plunger was used to adjust the size of the tube to accommodate the mouse and the animal was held in place with a bung. A V-shaped groove was cut in the bung to allow the tail to hang free, the end of the tail was snipped off with a sharp pair of scissors. For the collection of sera droplets of blood were collected in haematocrit tubes which were

centrifuged and the serum retained and stored at -40°C until required.

2.8.2 White Blood Cell Counts (WBC)

50 ul of blood was collected from the tail and taken up into a white cell pipette. The blood was then quickly diluted to 1:20 in a white blood cell counting fluid (1% acetic acid, 1% methyl violet). After rapid mixing for 30 secs. the cells were placed on a standard haemocytometer and the numbers of cells per ul of blood calculated.

2.8.3 Differential Cell Counts

Blood smears were taken at the same time as the white blood cell counts and dried in air. They were then fixed for 10 mins. in methanol and stained for 30-45 mins. in Giemsa (see Appendix). After washing well with tap water the slides were dried and 100 cells counted per slide under oil immersion.

2.8.4 `Node' Prints

At autopsy, the spleen and mesenteric lymph nodes (MLN) were carefully removed and the excess fat trimmed off. Using a sharp pair of scissors the organs were cut in half and the cut surface was gently dabbed onto a methanol cleaned slide. The slides were then dried, fixed in methanol and stained with Giemsa as for the blood smears.

2.8.5 Organ Cellularity

The total number of viable cells in each secondary lymphoid organ (spleen and MLN) were assessed by the ability of the live cells to hydrolyse fluorescein diacetate (FDA, Sigma) producing free fluorescein. When viewed under an Olympus BHS microscope with an Olympus BH-RFL-W reflected light fluorescent attachment, the live fluoresce green.

A stock solution of FDA (5 mg/ml in acetone) was prepared and stored at -20°C. A working dilution of 1:50 in PBS was made up immediately prior to use. The number of viable cells was assessed after the addition of 1 part FDA (working solution) to 9 parts cell suspension, and counted using a standard haemocytometer.

2.8.6 Assays for Blast Cell Activity/Responses to Mitogen

Spleens and MLN were removed from infected animals at a range of times post infection (p.i.) or post challenge along with age matched control animals. The lymphocyte proliferation assay was basically that described by Wakelin, Mitchell, Donachie and Grencis (1985). Briefly, organs from individual animals within groups were pooled and used to produce cell suspensions to a final concentration of 1 x 10^6 ml. 50 ul aliquots were delivered into flat bottomed, 96 well tissue plates (Nunclon). For the assessment of the response to mitogens concanavalin A (Con A) and lipopolysaccharide (LPS), 50 ul aliquots of solutions of known concentration were added to the appropriate cells in triplicate. Blast cell activity was assessed following incubation with medium alone (see Appendix). All wells, including the appropriate controls, were made up to a final volume of 200 ul with medium. Titrations of Con A and LPS allowed optimal doses of mitogen (20 ug/ml) to be used. All washings and dilutions were carried out with medium containing 10% foetal calf serum (see Appendix). The plates were then incubated at 37°C (5% CO_2, 95% air) for 24 hours. The degree of proliferation was assessed by pulsing the cells with ^{3}H-thymidine (^{3}H-Tdr, Amersham). 0.5 mCi ^{3}H-thymidine was added, in 50 ul, to each well and incubated for a further 24 hours. Cells were harvested onto glass fibre discs using a Dynatech Multimash 200 cell harvester, dried and then placed into 5 ml of scintillation fluid (Optiphase 'X', Fisons) in capped vials (Hughes and Hughes). The amount of radio-activity in each disc was measured using a Packard Tricarb liquid scintillation spectrophotometer.

2.8.7 Assessment of Numbers of T and B Cells

The numbers of T and B cells in the spleen and MLN were assessed using fluoroisothiocyanate (FITC) conjugated antibodies. Cells with bound antibody fluoresce green when viewed under a Olympus BHS microscope, with a Olympus BH-RFL-W reflected light fluorescent attachment. Briefly, the numbers of B cells were assessed by incubating 5 x 10^6 cells in 50 ul of an FITC conjugated rabbit anti-mouse polyvalent immunoglobulin (Sigma) at a dilution of 1:20 in medium containing 10% FCS and 1% heat inactivated normal rabbit serum in order to block Fc binding. The numbers of T cells were measured by incubating 5 x 10^6 cells in 50 ul of a rat anti-mouse Thy-1 cell marker (SeraLab) at a dilution of 1:500 in the same medium. Following incubation for 30 mins on ice, the cells were washed three times with medium. The B cells were immediately counted on a standard haemocytometer, both the labelled and unlabelled cells were counted and the proportion with bound antibody assessed. The T cells were further incubated in a FITC conjugated goat anti-rat IgG (Sigma) for 30 mins. on ice, washed three times and counted as for the B cells. A control for the T cells was also counted, this consisted of cells which were only incubated with the conjugate.

2.9 Histological Techniques

2.9.1 Preparation of Fixed Intestinal Sections

At autopsy, 1 cm pieces of small intestine were removed from approximately 1 cm below the ileo gastric junction. These were laid onto filter paper and immersed in formol buffered saline (see Appendix).

Fixed tissues were placed in wire baskets and dehydrated using a Hendrey Relay Histokinette. The histological processor passed the tissues through 6 changes of ethanol (70%, 80%, 90%, 95% and 2 x 100%) followed by 3 changes of xylene and finally a

paraffin polymer (Polywax, Difco Ltd.) at 56°C, remaining in each solution for one hour. The tissues were embedded in the paraffin polymer, mounted on wooden blocks and 5 um sections were cut using a Jung microtome.

2.9.2 Staining of Sections for Mast Cells

Cut sections were de-paraffinised and rehydrated by the following procedure: sections were heated to 56°C followed by 5 mins in each of 2 xylene washes and 2 100% ethanol washes followed by 2 mins in each of 90%, 80%, 70% and 50% ethanol and finally one dip in water. Sections were then stained as follows (see Appendix for recipe of stains). The slides were placed in Alcian/Astra Blue (1:1 v/v), preheated to 56°C for 1 hour. The slides were quickly rinsed in 0.7 N HCl and the tissues counterstained by immersion in pre-warmed (56°C) Safranin O for 3 mins. The slides were then dehydrated by rapidly dipping in each of distilled water, 70%, 90% and 2 x 100% ethanol followed by immersion for 1 min. in absolute ethanol:xylene (1:1 v/v). Clearing was accomplished by 3 x 3 mins. in fresh xylene, the slides were then mounted in DePeX. 25 villus crypt units were counted from 5 sections per animal.

2.10 Drug Treatments

2.10.1 Piperazine

This drug was used in order to reduce the incidence of pinworm in mice. A 12.5% solution in distilled water of piperazine citrate was made up and 0.2 ml was administered orally via a blunt ended 21 G needle. This was repeated 1 week later and then the mice were used 1 week after this.

2.10.2 Pyrantel

Pyrantel embonate (Strongoid-P, Pfizer) was used at a dose of 100 mg/kg to paralyse adult *H. polygyrus in situ*. This dose has been shown (Behnke and Wakelin, 1977) to be totally effective at removing the parasite from the host. The drug was suspended in distilled water and administered orally via a blunt ended 21G needle.

2.10.3 Terramycin

Terramycin (oxytetracycline hydrochloride, Pfizer) was administered at 165 ug/l in the drinking water of mice showing the symptoms of bacterial infections.

2.11 Statistical Analysis of Results

Results were generally presented as mean values (x) plus or minus the standard error of the mean (SEM) for groups of animals undergoing uniform treatment. Differences between grouos were analysed using the Students t-test corrected for multiple comparison (Sokal and Rohlf, 1969). In all cases a probability value (p) less than or equal to 0.05 was considered significant.

2.12 APPENDIX

2.12.1 General Solutions

(a) Phosphate Buffered Saline (PBS) pH 7.4

NaCl	8.0g
KCl	0.2g
$Na_2HPO_4 12H_2O$	1.15g
KH_2PO_4	0.2g

The pH was adjusted to pH 7.4 with NaOH and made up to a final volume of 1 l with distilled water.

(b) TRIS Buffered Saline (TBS) pH 7.6

NaCl	9.0g
TRIS	6.21g

The pH was adjusted to pH 7.6 with HCl and made up to a final volume of 1 l with distilled water.

(c) Hanks' Saline (Modified Hanks' Balanced Salt Solution)

Hanks' solution was modified by excluding glucose and $NaHCO_3$ and increasing the remaining salts to an osmotic pressure of 300 mosmole.

Solution 1

NaCl	168.0g
KCl	8.0g
Na_2HPO_4	2.0g
KH_2PO_4	4.0g
0.2% phenol red	200ml

Make up to 2 l with distilled water.

Solution 2

$CaCl_2 . 2H_2O$	3.92g
$MgCl_2 . 2H_2O$	2.0g

Make up to 2 l with distilled water.

110 ml of solution 1 were added to 110 ml of solution 2 and 780 ml of distilled water. The pH was adjusted to pH 7.2 with 1 M NaOH.

(d) 40% Zinc Sulphate Solution (Faecal Egg Counts)

$ZnSO_4$	40g

Dissolved in 100 ml of distilled water.

2.12.2 Cell Culture Medium

RPMI 1640 Medium Plus Supplements

RPMI 1640 powdered medium	10.42g/l	(Gibco)
Sodium bicarbonate	2.0g/l 20mM	
Glutamine	2mM	(Gibco)
Sodium pyruvate	0.1mM	(Gibco)
HEPES	10mM	(Gibco)
Monothioglycerol	7.5×10^{-5}	(Sigma)
Penicillin/Streptomycin		
penicillin	100 units/ml	(Gibco)
streptomycin	100ug/ml	
Foetal calf serum	100ml/l	(Serotech)

Medium was made up with double distilled de-ionised water, prepared in 5-10 l batches and filter sterilised and stored at $4^{o}C$ until use.

2.12.3 Stains and Fixatives

(a) Formol Saline

NaCl	4.5g
Formalin	50ml
Distilled water	450ml

(b) Astra Blue 1% pH 1.0

Astra Blue	1.0g (Gurr)
0.7N HCl	100ml

(c) Alcian Blue 1% pH 0.3

Alcian Blue	1.0g (Gurr)
0.7N HCl	100ml

(b) and (c) were used at a ratio of 1:1.

(d) Safranin 0.5% pH 1.0

Safranin	0.5g (Gurr)
0.125N HCl	100ml

(e) Giemsa

Sorenson's Buffer pH 6.8

Sorenson's Stock	0.5ml
Distilled water	99.5ml

Mix and pH adjust to 6.8 with 1M HCl.

Giemsa stain

May Grunwald and Giemsa Stain	10ml (Gurr)
Sorenson's Buffer pH 6.8	90ml

Make up fresh each time.

2.12.4 Solutions for Lowry Protein Estimation

Working Reagent

Solution 1 (made fresh)

Na_2CO_3	5.0g
NaOH	1.0g
Distilled water	250ml

Solution 2

$CuSO_4$	1.0g
Distilled water	100ml

Solution 3

NaKTartrate	2.0g
Distilled water	100ml

Working reagent consists of 50ml of solution 1 plus 1ml of each of solutions 2 and 3.

2.12.5 Solutions and Buffers for AChE assay

(a) Phosphate Buffer 0.1M pH 7.0 and pH 8.0

Solution 1

$NaH_2PO_4.H_2O$	13.8g
Distilled water	1.0l

Solution 2

Na_2HPO_4	14.2g
Distilled water	1.0l

Stock solutions are prepared and stored at 4°C. The two solutions are mixed to obtain the required pH.

(b) Reagent (Dithiobisnitrobenzoic acid, DTNB)

DTNB	0.396g
$NaHCO_2$	0.15g
Phosphate Buffer pH 7.0	100ml

(c) Substrates

Acetylthiocholine (ACT)

ACT	2.17g
Phosphate Buffer pH 8.0	100ml

Butrylthiocholine (BCT)

BCT	2.38g
Phosphate Buffer pH 8.0	100ml

2.12.6 ELISA Solutions and Buffers

(a) 0.05M Carbonate/Bicarbonate pH 9.6

Na_2CO_3	1.59g
$NaHCO_3$	2.93g
NaN_3	0.2g

Made up to 1 l with distilled water and pH adjusted.

(b) Diethanolamine Buffer pH 9.8

$MgCl_2.6H_2O$	0.101g
Diethanolamine	97ml

Made up to 1 l with distilled water and pH adjusted with 1M HCl.

(c) PBS-Tween

Tween 20	0.5ml

(polyoxyethylene sorbitan monolaurate)

Made up to 1 l with PBS.

(d) ELISA Substrate

Tablets of p-nitrophenyl phosphate (Sigma) were stored at -20°C and freshly made up into a working solution each time the assay was used. One tablet dissolved in 5 ml of diethanolamine buffer and kept dark.

2.12.7 SDS-PAGE Solutions and Buffers

(a) Electrode Buffer (0.25M TRIS pH 8.3)

TRIS	15.15g
Glycine	72.0g
SDS (Sodium dodecyl sulphate)	5.0g

Dissolved in 200ml distilled water, pH adjusted with HCl and made up to 5 l with distilled water.

(b) Resolving Gel Buffer (3.0M TRIS pH 8.8)

TRIS	90.5g
SDS	2.0g

Dissolved in 200ml distilled water, pH adjusted with HCl and made up to 500ml with distilled water.

(c) Stacking Gel Buffer (0.5M TRIS pH 6.8)

TRIS	30.2g
SDS	2.0g

Dissolved in 200ml distilled water, pH adjusted with HCl

and made up to 500ml with distilled water.

(d) Stock Acrylamide/Bis Acrylamide Solution

Acrylamide	30.0g
N,N'-methyl bis acrylamide	0.8g

Made up to 100ml with distilled water.

(e) 5% Acrylamide Solution

Stock acrylamide:bis (30:0.8)	5.0ml
Resolving gel buffer	3.75ml
10% SDS	0.3ml
Distilled water	20.25ml

(d) 20% Acrylamide Solution

Stock acrylamide:bis	20.0ml
Resolving gel buffer	3.75ml
10% SDS	0.3ml
Distilled water	2.75ml

(e) Substrate Gel

Stock acrylamide:bis	32.0ml
1.5M Tris pH 6.8	20.0ml
10% SDS	0.8ml
Distilled water	27.0ml
10% evaporated milk	8.0ml

(f) Stacking Gel

Stock acrylamide:bis	1.3ml
Stacking gel buffer	2.5ml
10% SDS	0.1ml
Distilled water	6.1ml

(g) 10% Ammonium Persulphate

1g APS in 10ml distilled water, made up fresh each time.

(h) Reducing Sample Buffer

0.5M TRIS (pH 6.8)	12.5ml
SDS	2.3g
Glycerol	10.0ml
B-ME (B-mercapto ethanol)	5.0ml
1% BPB (Bromophenol blue)	1.0ml (Gurr)

Made up to 100ml with distilled water.

(i) Non-Reducing Sample Buffer

As for reducing sample buffer but excluding the B-ME.

(j) Coomassie Stain

Coomassie Brilliant Blue R250	2.5g
Methanol	400ml
Acetic acid	100ml
Distilled water	500ml

Dissolve and filter before use.

(k) Destain

Methanol	400ml
Acetic acid	100ml
Distilled water	500ml

(o) 2.5% Triton X 100

Triton X 100	2.5ml

(Octyl phenoxy polyethoxyethanol, Sigma)

Distilled water	97.5ml

(p) ST Buffer

1% SDS in 0.125M TRIS pH 6.8

(q) ST Buffer containing 5% B-ME

ST buffer	95ml
B-ME	5.0ml

(r) 0.25% CTAB

0.25g CTAB (cetyltrimethylammonium bromide) in 100ml of Eagles MEM.

(s) Triton X 114 Buffer pH 7.4

Triton X 114	1ml
NaCl	0.89g
TRIS	0.121g
Distilled water	100ml

Adjust pH to 7.4 with 1M HCl.

(t) Triton X 114 Sucrose Cushion

Triton X 114	0.06ml
Sucrose	6.0g
NaCl	0.89g
TRIS	0.121g
Distilled water	100ml

Adjust pH to 7.4 with 1M HCl.

2.12.8 Western Blot Solutions and Buffers

(a) Blotting Buffer

TRIS	12.0g
Glycine	57.6g
Methanol	1l

Made up to 5 litres with distilled water.

(b) Blocking Buffer (10%)

Skimmed milk Marvel)	20g
PBS-Tween	200ml

(c) Peroxidase Substrate

4-chloro-1-napthol	30mg
Absolute ethanol	1ml

Added, while stirring, to 100ml of TBS pH 7.6. Next, 1ml of 3% hydrogen peroxidase was added and the mixture filtered into a dark bottle. Must be made up fresh.

(d) Amido Black Stain

Amido Black	0.25g
Methanol	400ml
Acetic acid	100ml
Distilled water	500ml

Filter before use.

2.12.9 Immunoprecipitation Buffer

Triton X 100	50ul
TRIS (2M solution pH 8.0)	2.5ml
NaCl	0.87g
EDTA (10mM solution)	2.0ml

Mix and pH to 7.4.

2.12.10 Autoradiography Solutions

(a) Developer

1:6 AGFA Gevaert (G150) in tap water.

(b) Fixer

1:5 AGFA Gevaert (G350) in tap water.

CHAPTER THREE

STAGE SPECIFIC EXPRESSION OF FUNCTIONAL ENZYMES OF *HELIGMOSOMOIDES POLYGYRUS*

3.1 INTRODUCTION

The stage specific proteins of *Heligmosomoides polygyrus* were investigated in two ways; functional enzymes in both homogenates (somatic proteins) and ES (excretory/secretory products) were analyzed and the structural or cuticular proteins were examined using radio-iodination techniques. This chapter concentrates on the stage specific production of functional enzymes, particularly in the ES, and their possible importance in the development of immunity to the various stages of the parasite. The next chapter discusses the role of stage specific cuticular proteins.

The release of antigenic material during *in vitro* culture has been widely demonstrated for a great range of parasitic nematodes such as *Trichuris muris* (Jenkins and Wakelin, 1983), *Trichinella spiralis* (Silberstein and Despommier, 1985a,b), *Toxocara canis* (Nicholas, Stewart and Mitchell, 1984; Badley, Grieve, Rockey and Glickman, 1987), *Ascaris suum* (Urban and Romanowski, 1985; Kennedy and Qureshi, 1986) and *Necator americanus* (Carr and Pritchard, 1986). It was also documented that immunisation with crude ES material could induce partial protection of the host to a homologous challenge infection. In *T. spiralis* it has been shown that antigens from the ES, that were derived from the stichosome, could induce a protective response (Silberstein and Despommier, 1984, 1985a,b; Gamble, 1985). It was also thought that these molecules released *in vitro* are also released *in vivo* as they have been detected in host muscle cells containing the encysted larvae (Pritchard, Crawford, Duce and Behnke, 1985).

The ES products of helminths are known to exert a spectrum of influences on the immune responses of their mammalian hosts, ranging from the induction of host protective immunity to providing mechanisms by which the parasites can evade the hosts responses. These molecules may be both shed from the surface of the parasite or be actively secreted from specialised secretory organs (Lightowler and Rickard, 1988). Therefore, it was thought that the investigation of the stage

specific release of these molecules may provide some insight as to why the adult stage of the parasite is claimed to be immunosuppressive whilst the larval stages of the parasite are thought to be immunogenic.

The host-protective activity of antibodies against larval *H. polygyrus* ES antigens may well account for the enhanced resistance observed in animals infected with radiation-attenuated larvae, anthelmintic abbreviated infections, multiple infections or ectopic injection of larvae and/or their products (Behnke and Robinson, 1985; Behnke and Wakelin, 1977; Enriquez, Cypess and Wassom, 1988; Ey, 1988a,b; Goven and De Buysscher, 1980; Hagan, Behnke and Parish, 1981; Hosier, Sackman and Idell, 1974; Jones and Rubin, 1974; Mitchell and Munoz, 1983; van Zandt, 1973). The prolonged growth or injection into an ectopic site may result in a prolonged release of stage specific larval ES antigens, leading to increased titers to these molecules and enhanced resistance. Indeed, whilst van Zandt (1971) reported a 30% reduction in challenge infections in mice vaccinated with soluble extracts of adults, slightly better levels (50%) were obtained with vaccines from soluble extracts of infective larvae (Cypess, 1970b; Goven and DeBuysscher, 1980). Both adults and larvae implanted ectopically confer protection against challenge infection (Lueker, Rubin and Anderson, 1968; Rubin, Lueker, Flom and Andersen, 1971; Hurley, Day and Mitchell, 1980). Although the most impressive result was reported by Mitchell and Munoz (1983) who induced a greater than 95% protection in genetically susceptible C57BL/6 against *H. polygyrus* with soluble extracts of adult worm with pertussigen as adjuvant, the degree of developmental retardation and stunting of adult worms could be correlated with the titer of antibodies specific for larval ES antigens, but not with antibodies reacting with cuticular or internal somatic antigens (Ey, 1988a,b).

3.2 RESULTS

3.2.1 Parasite Growth

In order to assess the effect of *in vitro* culture on the various stages of the parasite, the length of the worms before and 96 hours after culture for the collection of ES products was measured. It was noted (Fig. 3.1) that the mean length of the worm did not appear to be affected by 96 hours *in vitro* culture. It was also obvious that that the parasite did not appear to increase substantially in size until day 4 post infection (p.i.), after which there was a steady growth of the parasite at least until 120 days p.i. The length of each sex is only shown from day 6 onwards as it was very difficult to differentiate between the sexes before this time. It was also pertinent to note that the majority of growth between days 6 and 120 p.i. was accounted for by the increase in the size of the female parasite, and not by that of the male worm, which did not appear to grow at all after day 6.

Figure 3.1 Growth of Parasites

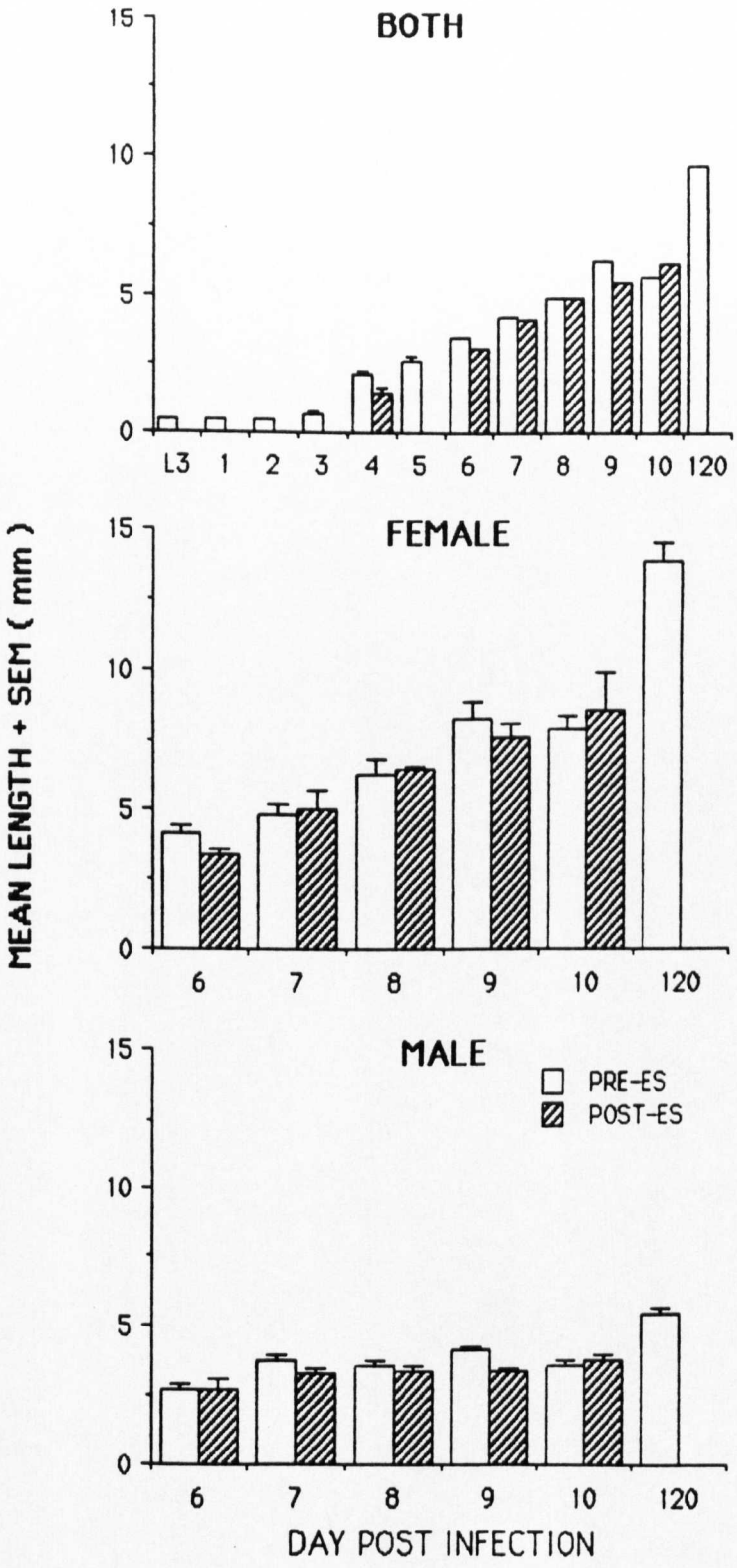
Parasites from L3 to day 120 p.i. were measured pre- and post-culture for ES products. The worms were drawn with the aid of a camera lucida and the lengths calculated with a bit pad digitiser linked to an Apple PC.

PRE-ES

Day 1 vs L3: NS
Day 2 vs Day 1: $p < 0.05$
Day 3 vs Day 2: $p < 0.005$
Day 4 vs Day 3: NS
Day 5 vs Day 4: $p < 0.05$
Day 6 vs Day 5: $p < 0.05$
Day 7 vs Day 6: $p < 0.05$
Day 8 vs Day 7: NS
Day 9 vs Day 8: $p < 0.05$
Day 10 vs Day 9: NS
Day 120 vs Day 10: $p < 0.005$

All Pre-ES vs Post-ES: NS

N.B. NS (not significant)


BOTH
FEMALE
MALE
PRE-ES
POST-ES
MEAN LENGTH + SEM (mm)
DAY POST INFECTION
15
10
5
0
L3
1
2
3
4
5
6
7
8
9
10
120

3.2.2 Parasite Culture

It has been demonstrated by other workers (Pritchard, Leggett, McKean and Rogan, unpublished observations) that culturing parasites in a diphasic medium, on a collagen raft, increased the protein and enzyme production and longevity of the parasites compared with those cultured in the absence of this insoluble base.

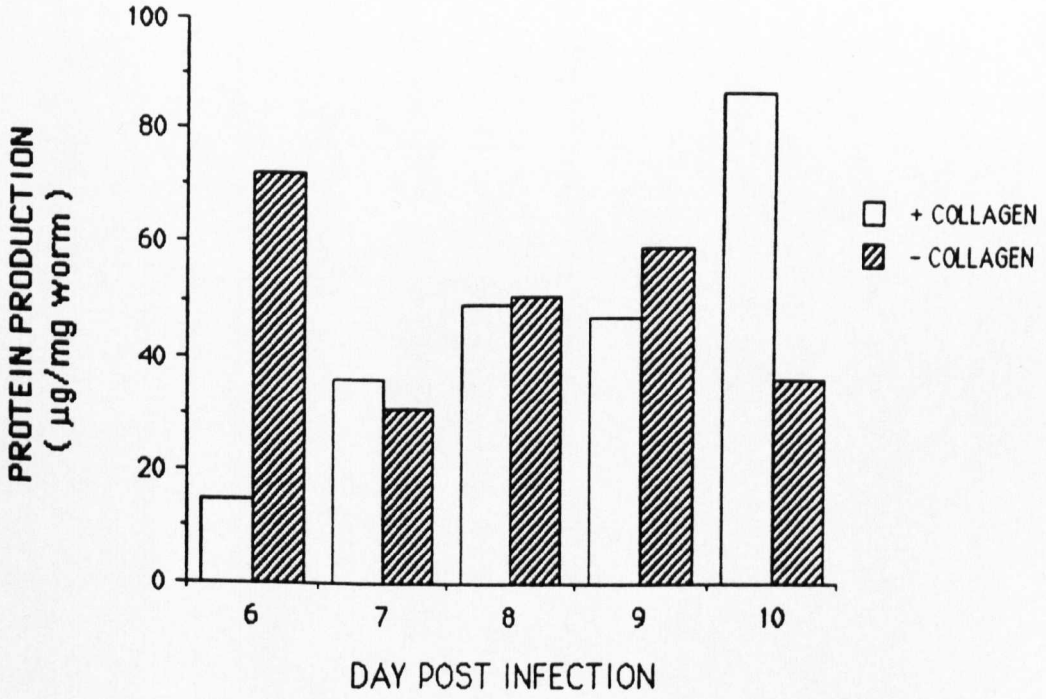
In order to maximize the protein production of the various stages of this parasite the worms were cultured both with and without a collagen raft. Only parasites between days 6 and 10 p.i. were used as insufficient material was obtained from the earlier stages. It can be seen (Fig. 3.2) that there was no apparent difference between the protein production of the two culturing methods. Whilst the production by day 10 appeared greater on the collagen rafts, that of day 6 was much decreased, with no observable differences on days 7-9.

This data represents ES from parasites pooled from 10 mice per group. The number of animals (100) and infectivve larvae (500,000) necessary for this experiment precluded further repeats and so no statistical analysis can be performed.

Figure 3.2 Effects of Culture Conditions on Protein Production

The protein production of stages of the parasite from day 6-10 p.i. was assessed when the worms were cultured for 96 hours both in the presence and the absence of collagen rafts. The amount of protein produced is expressed in terms of ug of ES produced per mg worm (wet weight).

This data represents ES from parasites pooled from 10 mice per group. The number of animals (100) and infectivve larvae (500,000) necessary for this experiment precluded further repeats and so no statistical analysis can be performed.


PROTEIN PRODUCTION
(µg/mg worm)
100
80
60
40
20
0
6
7
8
9
10
DAY POST INFECTION
+ COLLAGEN
- COLLAGEN

In order to assess the viability of the parasites in the two culture systems further, the acetylcholinesterase (AChE) and pseudocholinesterase (PChE) levels of the parasite were analyzed. It has been shown previously (Burt and Ogilvie, 1975) that the production of these enzymes was a marker of the rate and duration of antigen synthesis and secretion *in vivo*. The enzyme is also widely used as a parameter in the interpretation of *in vitro* anthelmintic screens (Rapson, Chilwan and Jenkins, 1986; Sangster, Pritchard and Lacey, 1985) and thus its level of production gives an indication of the parasite's functional status. There appears to be no increased viability of the parasites cultured on collagen rafts (Fig. 3.3), in fact, there even appears to be a slight decrease in the production of enzymes in the early stages of the parasite compared to culture in the absence of collagen.

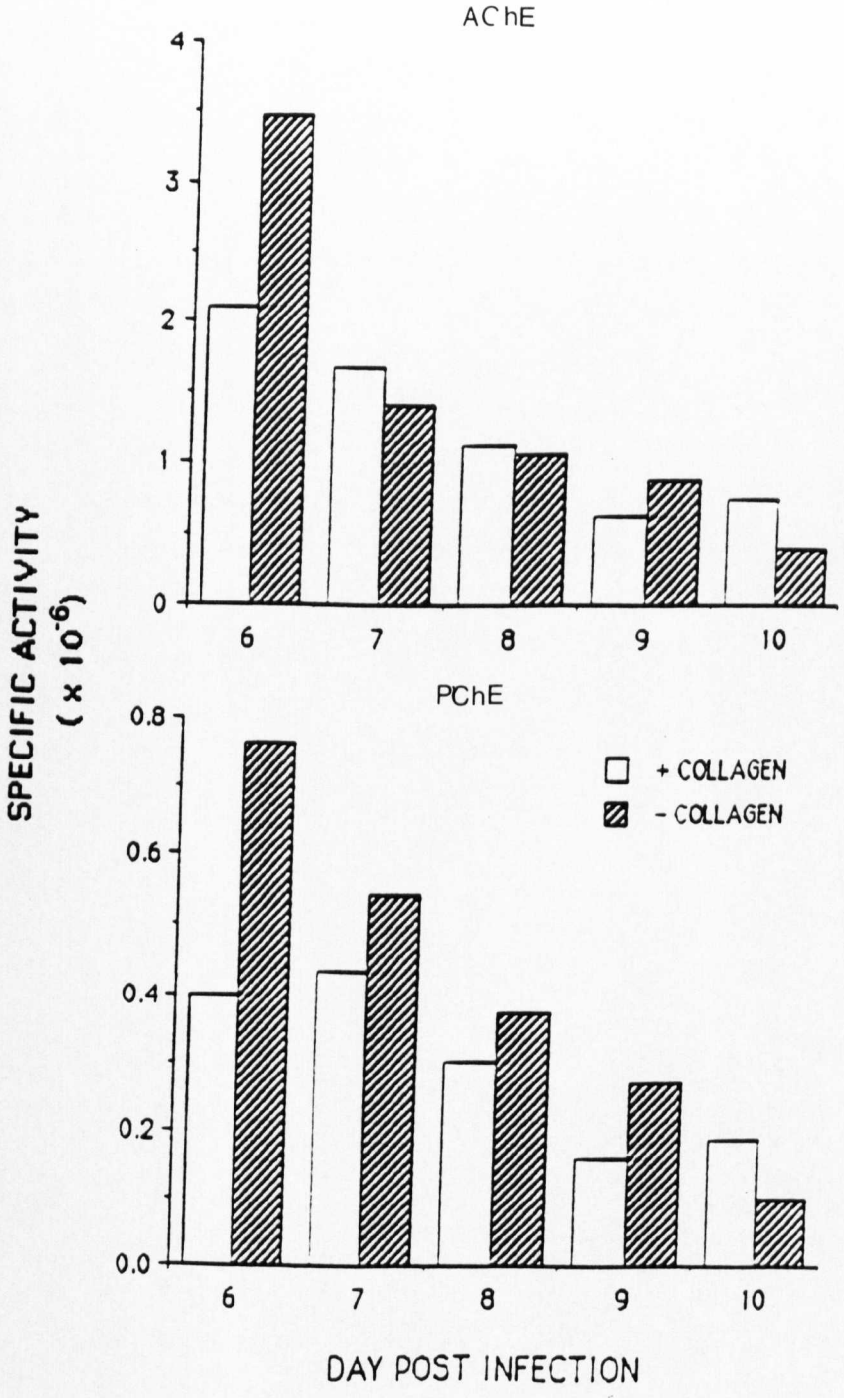
As a consequence of the results of this study it was decided to concentrate on culturing the parasites in a monophasic medium. Not only does this remove any extraneous protein released from the collagen matrix, but it is also quicker and easier to maintain.

This data represents ES from parasites pooled from 10 mice per group. The number of animals (100) and infectivve larvae (500,000) necessary for this experiment precluded further repeats and so no statistical analysis can be performed.

Figure 3.3 Effects of Culture Conditions on Enzyme Production

The acetylcholinesterase (AChE) and pseudocholinesterase (PChE) production of parasites between days 6 and 10 p.i. was assessed when the parasites were cultured for 96 hours both in the presence and the absence of collagen rafts. The activity is expressed in terms of specific activity (mol/l/min/mg parasite protein).

This data represents ES from parasites pooled from 10 mice per group. The number of animals (100) and infectivve larvae (500,000) necessary for this experiment precluded further repeats and so no statistical analysis can be performed.


AChE
4
3
2
1
0
6
7
8
9
10
PChE
0.8
0.6
0.4
0.2
0.0
+ COLLAGEN
- COLLAGEN
SPECIFIC ACTIVITY
(x 10⁻⁶)
DAY POST INFECTION

3.2.3 Stage Specific Acetylcholinesterase Production

The AChE and PChE content of both ES and homogenates of the various stages of the parasite are shown (Fig. 3.4). It was seen that, on average, the AChE content of ES was about one third that of the homogenate per mg of protein produced. From both of these preparations it was noted that the levels of AChE rose gradually from the infective third stage larvae (L3) to day 3 p.i. and then increased dramatically on day 4. This level was sustained until day 6 in the ES and day 7 in the homogenate and then declined rapidly, until the levels at day 120 onwards were lower than the levels of the infective larvae. The levels of PChE showed a different pattern, in the ES they were negligible and showed no increase at any stage. However, in the homogenate, the levels were as great as those of AChE up to day 3, and as much as AChE ES on day 6, but this had declined to negligible amounts by day 9 and was comparable to the low levels of AChE on day 120.

This data represents ES from parasites pooled from 10 mice per group. The number of animals (100) and infectivve larvae (500,000) necessary for this experiment precluded further repeats and so no statistical analysis can be performed.

Figure 3.4 Stage Specific Production of AChE and PChE

(A)

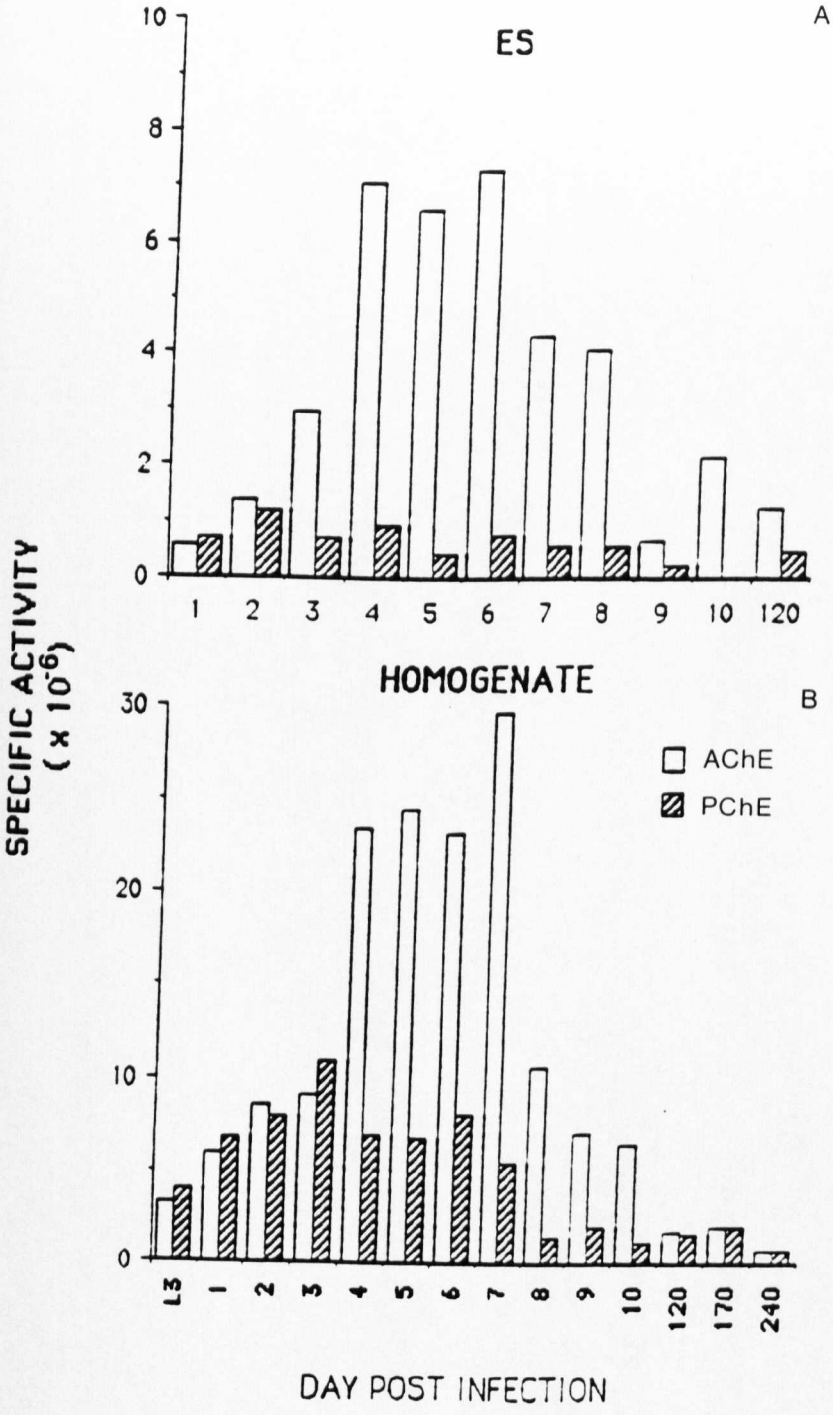
The content of AChE and PChE in the excretory/secretory (ES products) of various stages of the parasite was assessed for parasites cultured for 96 hours in the absence of collagen rafts.

(B)

The content of AChE and PChE in the homogenates of various stages of the parasite was assessed.

The activity is expressed in terms of specific activity (mol/l/min/mg parasite protein).

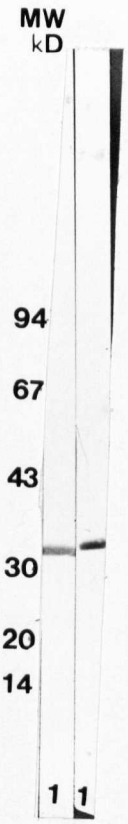
This data represents ES from parasites pooled from 10 mice per group. The number of animals (100) and infectivve larvae (500,000) necessary for this experiment precluded further repeats and so no statistical analysis can be performed.


A
ES
10
8
6
4
2
0
1 2 3 4 5 6 7 8 9 10 120
HOMOGENATE
B
AChE
PChE
SPECIFIC ACTIVITY
(x 10⁻⁶)
30
20
10
0
L3 1 2 3 4 5 6 7 8 9 10 120 170 240
DAY POST INFECTION

In order to try to characterise the AChE molecule further, a Western blot of day 10 adult ES antigens was probed using a serum which had been raised in rabbits against purified *Necator americanus* acetylcholinesterase (Pritchard, Leggett, McKean and Rogan, unpublished observations). The serum reacted against a molecule of approximate molecular weight 30.5 kDa, no other molecules were recognised (Fig. 3.5). No molecules were recognised by a normal serum control.

Figure 3.5 Immunoblot probed with anti-*N. americanus* AChE serum.

1 mg of day 10 ES was run under reducing conditions on a 5-20% SDS-PAGE gel. Following electrophoretic transfer to a sheet of nitrocellulose, the resulting immunoblot was cut into 5mm strips, probed with a rabbit anti-sera that had been raised against purified *N. americanus* AChE, and developed with HRP-Protein A. Values for molecular weights are shown on the left.


MW
kD
94
67
43
30
20
14
1
1

3.2.4 Stage Specific Protease Production

The role of parasite proteases in the development of the parasite was examined. Firstly, an attempt was made to try to quantify the amount of proteolytic enzymes present in both the ES and homogenate of the various stages of the parasite.

The method employed was very similar to that developed by Robertson, Kwan-Lim and Maizels (1988). In this case it was decided to use casein as the substrate rather than gelatin as this substrate had been previously used to assess the molecular weight profiles of protease enzymes of the various stages of the parasite (Lawrence and Pritchard, 1988). Fig. 3.6 shows the standard curve produced by incubating a range of concentrations of the enzyme trypsin with radio-labelled casein bound to polystyrene microtitre plates. This allowed the amount of casein digested by the parasite extracts to be expressed in terms of equivalent tryptic activity.

Figure 3.6 Standard Trypsin Curve

Various concentrations of trypsin in PBS pH 8.0 were added to the wells of a microtitre plate coated with radio-iodinated casein and the percentage released calculated. The values were plotted on a graph with a $\log_{10}$ scale on the x-axis.

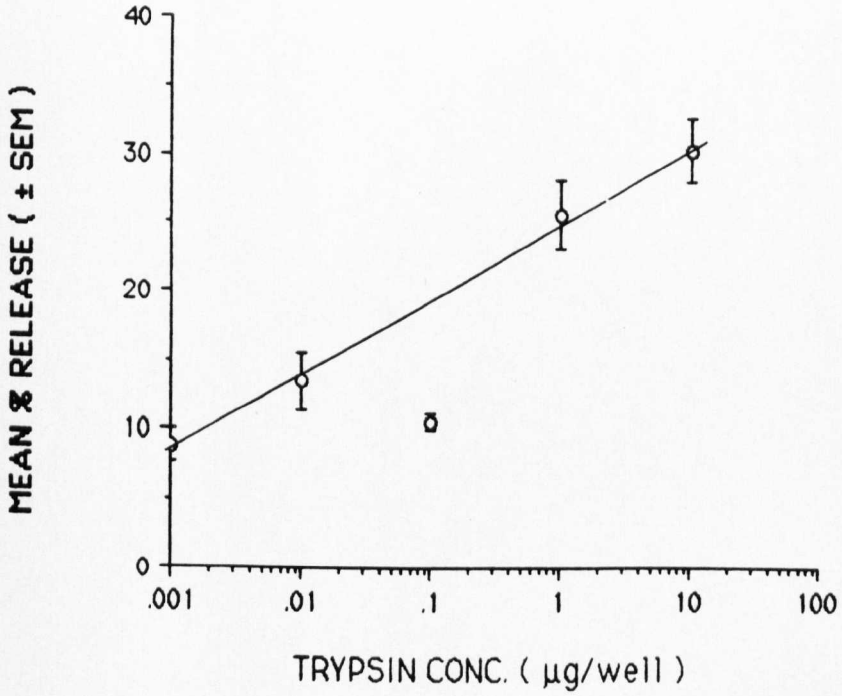

MEAN % RELEASE (± SEM)
40
30
20
10
0
.001
.01
.1
1
10
100
TRYPSIN CONC. (µg/well)

Fig. 3.7a, shows the mean percent release of casein from the plate by both parasite ES and homogenates. It was noted that that released by days 1-4 was greater than that released by the later stages and that the activity of the homogenates was comparable to that of the ES in the few preparations tested. When this activity was converted into tryptic units using the standard curve set up, the pattern was quite different (Fig. 3.7b). The activity of day 1 ES was about 20 times that of days 2-4, which had very similar activities, and about 100 times that of any of the other preparations. The reason for this different pattern in comparison to that obtained by the percentage release is due to the logarithmic nature of the trypsin standard curve.

Figure 3.7 Quantitative Analysis of Protease Activity

(A)

The mean percentage of radio-labelled casein released from coated microtitre plates by a range of preparations in PBS pH 8.0 was calculated and plotted.

(B)

The percentage of casein released by the parasite products was converted into equivalent trypsin activity using the standard curve and plotted. A tryptic unit is the equivalent of 1mg trypsin per mg parasite protein.

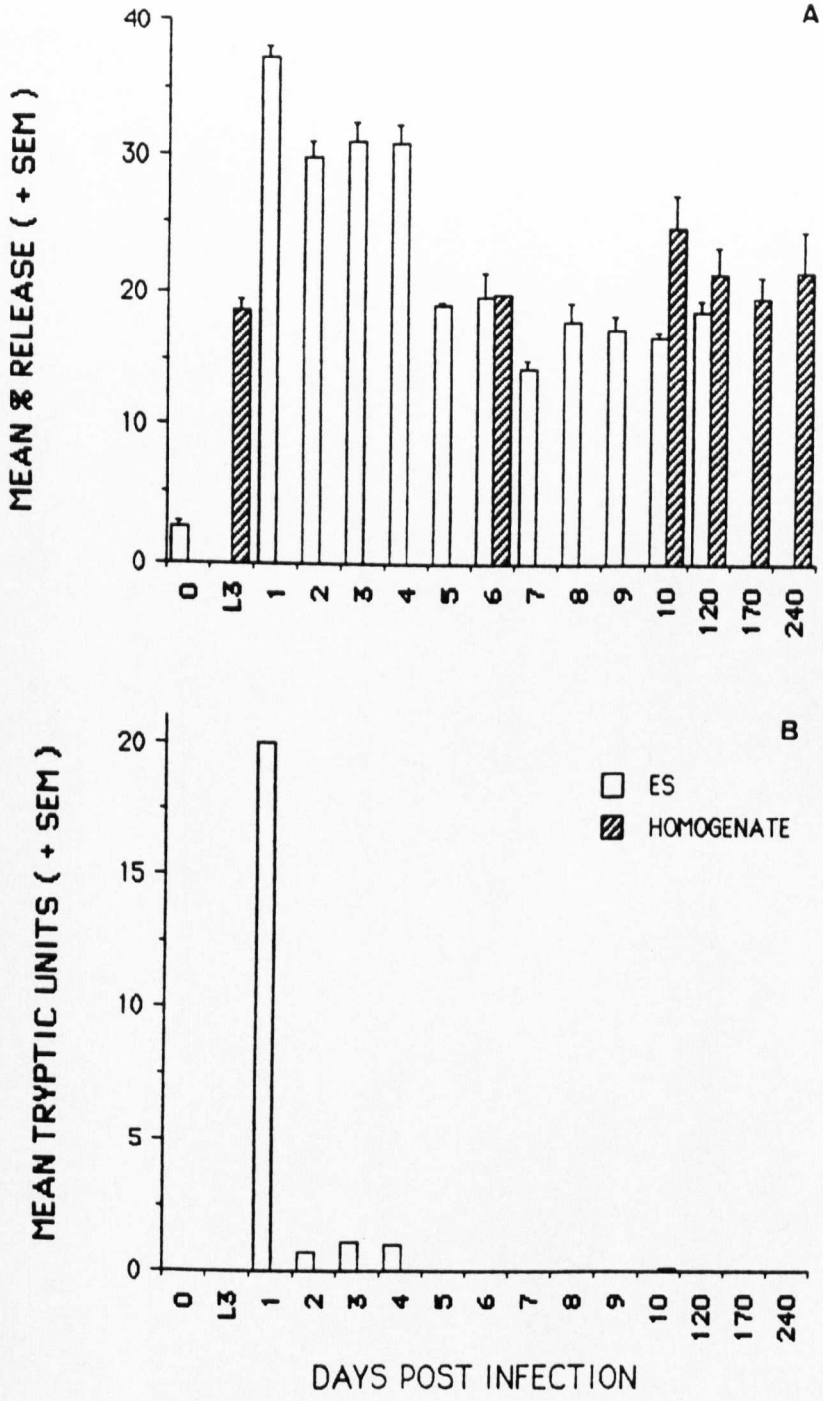

A
MEAN % RELEASE (+ SEM)
40
30
20
10
0
0
L3
1
2
3
4
5
6
7
8
9
10
120
170
240
B
MEAN TRYPTIC UNITS (+ SEM)
20
15
10
5
0
ES
HOMOGENATE
DAYS POST INFECTION

Table 3.1 gives the values of the activities obtained and it can be seen that although the amounts present in the later stages of the parasite were negligible in comparison to day 1, they were still quite substantial enough to cause degradation of the substrate.

Table 3.1 Quantitative Analysis of Protease Activity

The tryptic activity for a number of parasite preparations was calculated. A tryptic unit is the equivalent of 1mg trypsin per mg parasite protein.

DAY POST INFECTION	ES ACTIVITY (TRYPTIC UNITS ± SEM ($\times 10^3$))	HOMOGENATE ACTIVITY
L3	-	5.6 ± 2.82
1	20,000 ± 26.6	-
2	749 ± 3.16	-
3	1,122 ± 3.79	-
4	1,000 ± 2.76	-
5	6.7 ± 0.24	-
6	8.4 ± 0.22	10 ± 1.14
7	0.89 ± 0.25	-
8	4.2 ± 0.36	-
9	3.2 ± 0.32	-
10	2.2 ± 0.25	84.1 ± 5.3
120	5.6 ± 0.32	21.1 ± 0.45
170	-	8.9 ± 0.36
240	-	19.9 ± 0.71

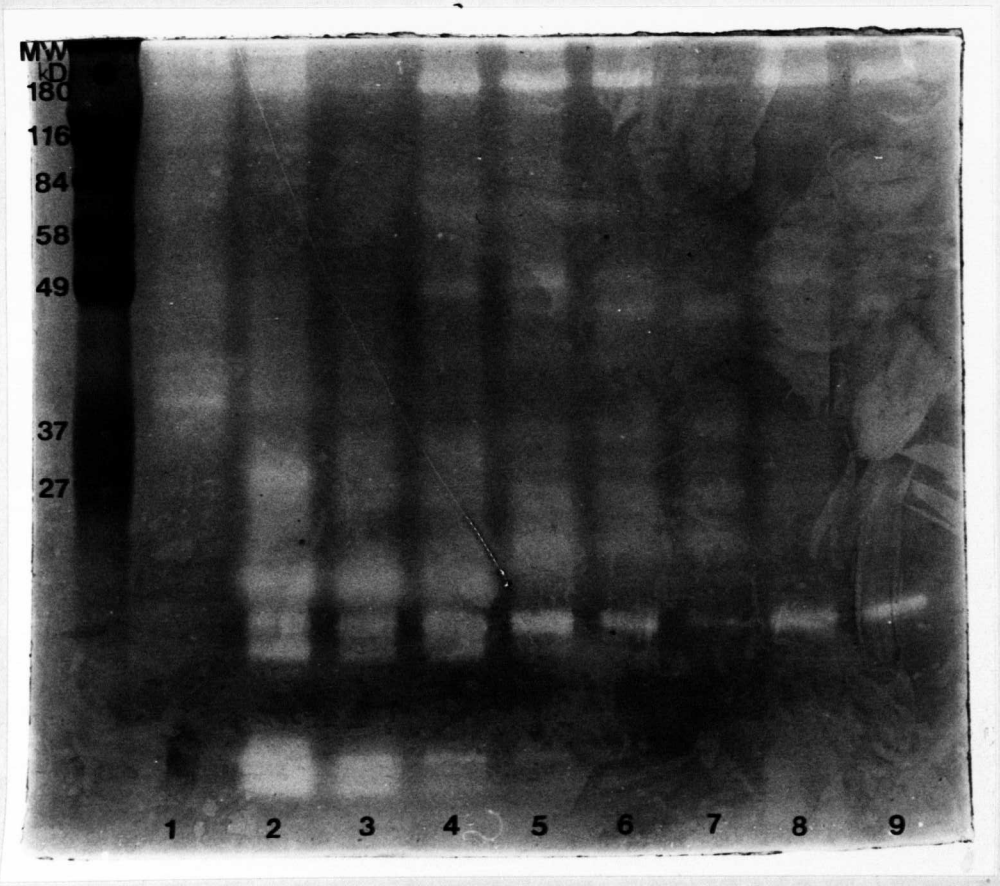
In order to qualitatively analyze the protease activity of the various preparations, samples were separated using casein substrate gels. Casein was used as the substrate rather than others such as gelatin as it has been shown that there is substantial activity using this substrate in both quantitative analyses (Fig. 3.7) and qualitative analyses (Lawrence and Pritchard, 1988). Fig. 3.8 shows that activity of the homogenates against casein (Carnation evaporated milk). The pale areas show the presence of enzyme activity. It was seen that L3 showed activity mainly at 96, 77, 68 35 and 30 kDa and that by day 1 p.i.there was also activity at 22, 15, 13, 12 and 8 kDa although that at 35 kDa had disappeared. By day 2 the molecules at 77, 68 and 30 kDa had gone but by day 3 proteases at 60 and 58 kDa had appeared with an additional molecule at 42 kDa being present by day 4. Day 5 saw the appearence of a molecule at 41 kDa concommittant with the absence of molecules at 60, 58, 15, 12 and 8 kDa. At the time of the second moult from L4 to adult a protease at 51 kDa appeared but that at 22 kDa disappeared. By day 9 a molecule at 116 kDa was present but this had gone by day 120. Very few molecules were seen past day 10 and these resolved at 102 and 13 kDa. A summary of the proteases present in parasite homogenates at the various time points post infection is illustrated in Table 3.2.

Figure 3.8 *In situ* Localisation of Proteases in Parasite Homogenates

Samples of 100ug of protein from PBS extracted homogenates of various stages of the parasite were run under non-reducing conditions on a 12% casein substrate gel. After electrophoresis, the gels were incubated for 24 hours in 0.1M glycine NaOH pH 8.0 at 37°C and then stained with Coomassie Brilliant Blue. The molecular weight standards are shown on the left hand side.

(A)	Lane 1	L3	(B)	Lane 1	Day 9
	" 2	Day 1		" 2	Day 10
	" 3	Day 2		" 3	Day 120
	" 4	Day 3		" 4	Day 170
	" 5	Day 4		" 5	Day 240
	" 6	Day 5			
	" 7	Day 6			
	" 8	Day 7			
	" 9	Day 8			

A


MW
kD
180
116
84
58
49
37
27
1
2
3
4
5
6
7
8
9

B

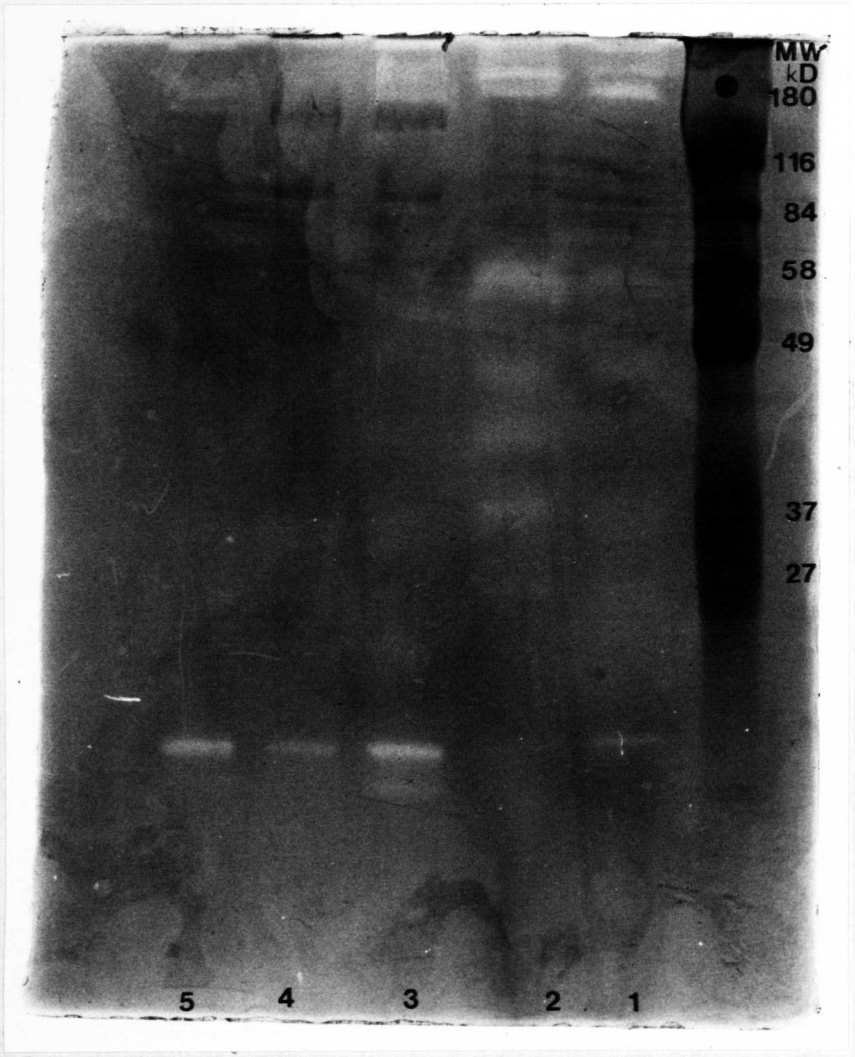

MW
kD
180
116
84
58
49
37
27
5
4
3
2
1

Table 3.2 Proteases Present in Parasite Homogenates

The major protease molecules present in homogenates of the various stages of the parasites were calculated from the protease gels and are summarised in the table.

Molecules Present (kDa)

Stage																			
L3	-	-	96	77	68	-	-	-	-	-	35	-	30	-	-	-	-	-	-
Day 1	-	-	96	77	68	-	-	-	-	-	-	-	30	-	22	15	13	12	8
Day 2	-	-	96	-	-	-	-	-	-	-	-	-	-	-	22	15	13	12	8
Day 3	-	-	-	-	-	60	58	-	-	-	-	-	-	-	22	15	13	12	8
Day 4*	-	-	-	-	-	60	58	-	42	-	-	-	-	-	22	15	13	12	-
Day 5	-	-	-	-	-	-	-	-	42	41	-	-	-	-	22	-	13	-	-
Day 6#	-	-	-	-	-	-	-	-	42	41	-	-	-	-	22	-	13	-	-
Day 7	-	-	-	-	-	-	-	51	42	41	-	-	-	-	-	-	13	-	-
Day 8	-	-	-	-	-	-	-	51	42	-	-	-	-	-	-	-	13	-	-
Day 9	116	102	-	-	-	-	-	51	42	-	-	-	-	-	-	-	13	-	-
Day 10	116	102	-	-	-	-	-	51	42	-	-	33	-	27	-	-	13	-	-
Day 120	-	102	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13	-	-
Day 170	-	102	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13	-	-
Day 240	-	102	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13	-	-

N.B. * Moult from L3 to L4
Moult from L4 to adult

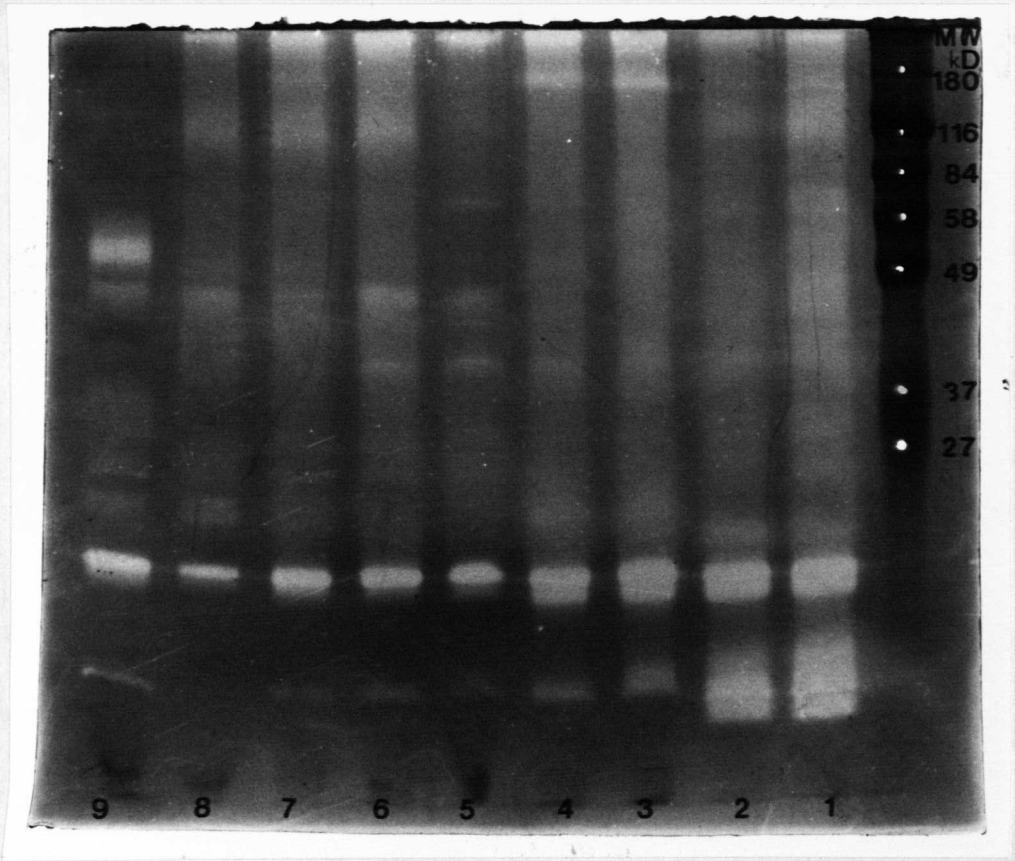
Fig. 3.9 illustrates the proteolytic activity of the ES products of various stages of the parasite. Overall, it was noted that the activity was much higher than in the homogenates. Again, there was activity at 96, 15, 13 and 8 kDa, but this seemed to decrease by day 4 rather than by day 3 as for the homogenate. On day 4 molecules at 76, 58, 42 and 33 kDa were apparent, those at 76, 42, 18, 16 and 13 kDa appeared to be present for the rest of the time period studied but those at 58, 33 and 15 kDa had all disappeared by day 7. By day 10 extra proteases were also apparent at 116, 102, 39 and 25 kDa. The molecules at 116, 102, 96, 58, 42, 15, 13 and 8 kDa seemed to be identical in both molecular weight and stage specific production as those seen in the homogenates of the parasite. Table 3.3 gives a summary of those parasite proteases present in the excretory/secretory products of the various stages of the parasite.

Figure 3.9 *In situ* Localisation of Proteases in Parasite ES Products

Samples of 100ug of protein from the ES products of various stages of the parasite cultured for 96 hours in the absence of collagen were run under non-reducing conditions on a 12% casein substrate gel. After electrophoresis, the gels were incubated for 24 hours in 0.1M glycine NaOH pH 8.0 at 37^{o}C and then stained with Coomassie Brilliant Blue. The molecular weight standards are shown on the left hand side.

(A)	Lane 1	Day 1	(B)	Lane 1	Day 10
	" 2	Day 2		" 2	Day 10 Hom.
	" 3	Day 3		" 3	Day 120
	" 4	Day 4		" 4	Day 120 Hom.
	" 5	Day 5		" 5	Day 170 Hom.
	" 6	Day 6			
	" 7	Day 7			
	" 8	Day 8			
	" 9	Day 9			

A


MW
kD
180
116
84
58
49
37
27
9
8
7
6
5
4
3
2
1

B

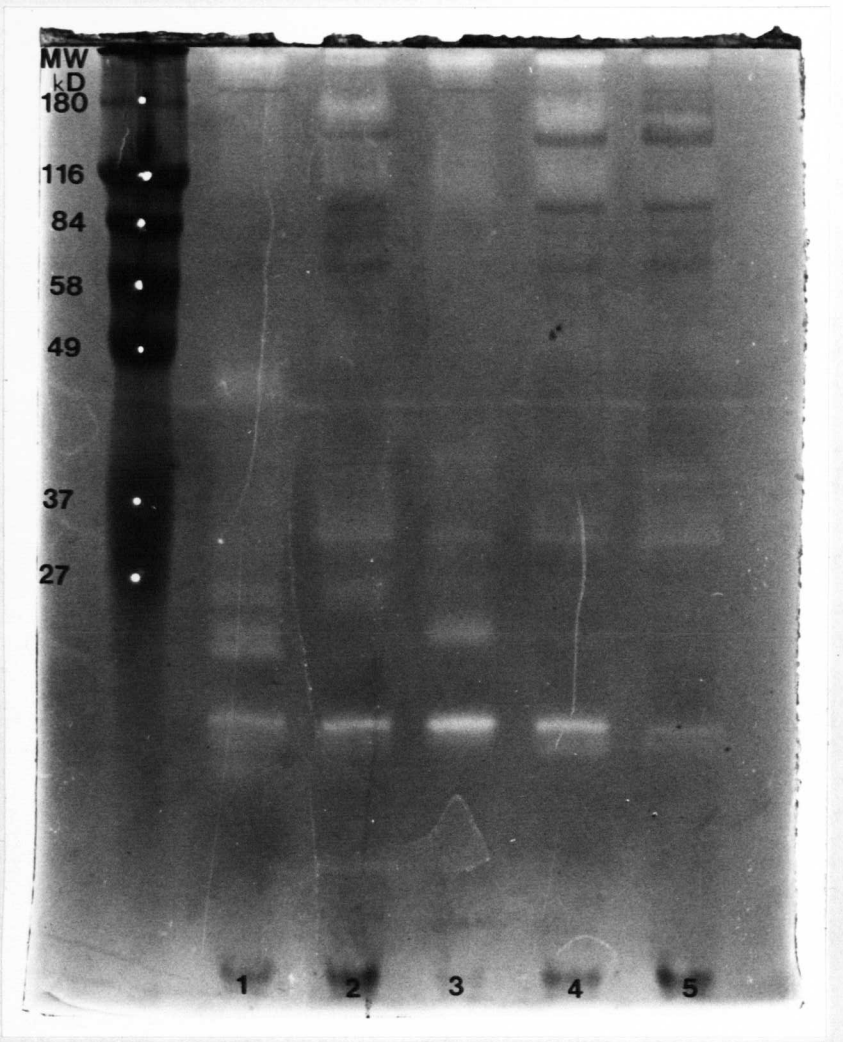

MW
kD
180
116
84
58
49
37
27
1
2
3
4
5

Table 3.3 Proteases Present in Parasite ES Products

The major protease molecules present in the ES products of the various stages of the parasites were calculated from the protease gels and are summarised in the table.

Stage	Molecules Present (kDa)																
Day 1	-	-	96	-	-	-	-	-	-	-	-	18	16	15	13	8	
Day 2	-	-	96	-	-	-	-	-	-	-	-	18	16	15	13	8	
Day 3	-	-	96	-	-	-	-	-	-	-	-	18	16	15	13	-	
Day 4*	-	-	96	76	58	-	-	-	33	-	-	18	16	15	13	-	
Day 5	-	-	-	76	58	-	42	-	33	-	-	18	16	-	13	-	
Day 6#	-	-	-	76	-	-	42	-	33	-	-	18	16	-	13	-	
Day 7	-	-	-	76	-	-	42	-	33	-	-	18	16	-	13	-	
Day 8	-	-	-	76	-	-	42	-	-	-	-	18	16	-	13	-	
Day 9	-	-	-	76	-	52	42	-	-	-	-	18	16	-	13	-	
Day 10	116	-	-	76	-	-	42	-	-	-	20	-	16	-	13	-	
Day 120	116	102	-	76	-	-	-	39	-	25	-	18	16	-	13	-	

N.B.* Moult from L3 to L4
\# Moult from L4 to adult

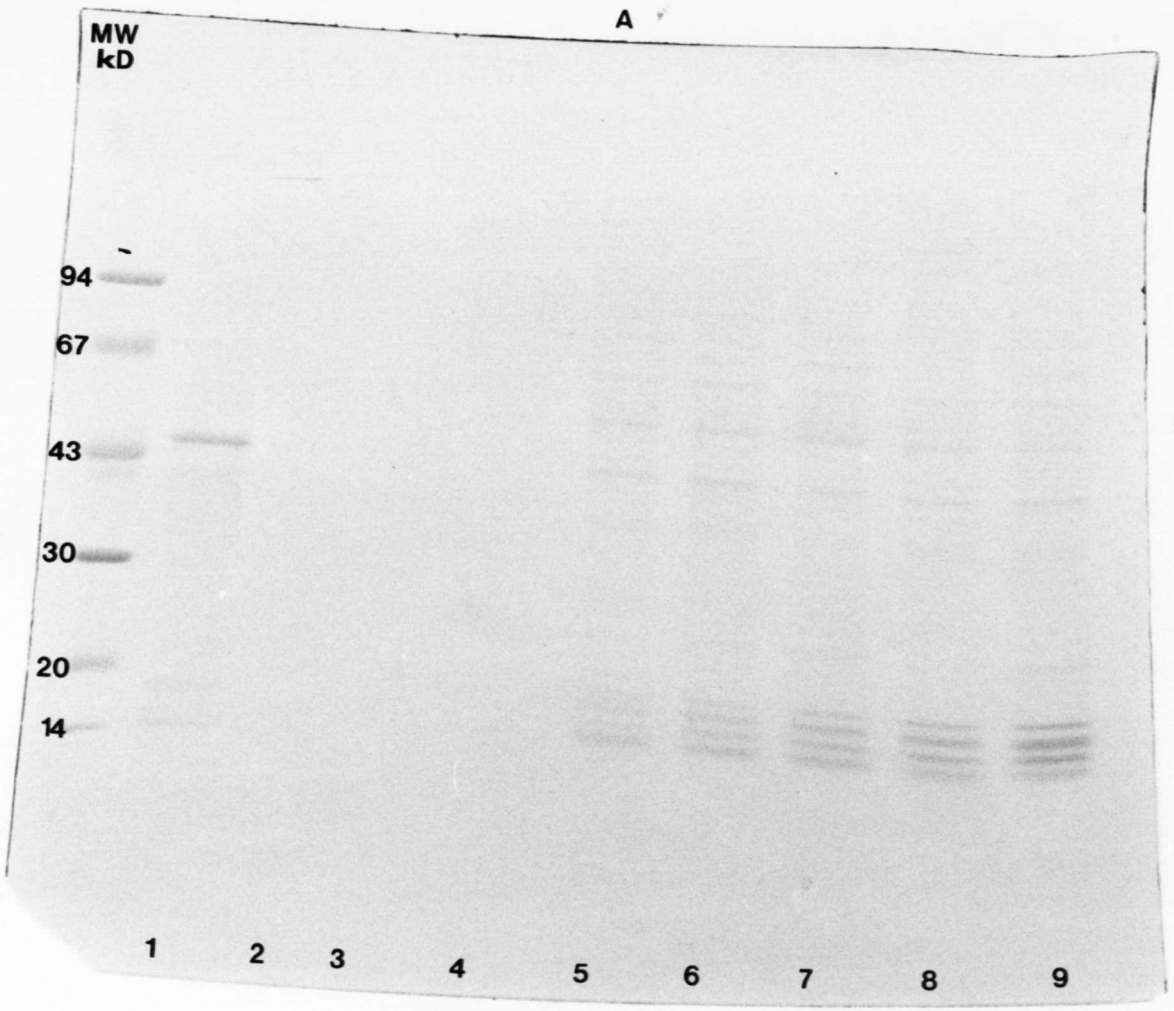
3.2.5 Stage Specific Protein Production

In order to try to correlate this activity with specific proteins and possibly an antibody response to them a protein gel was run and stained with Coomassie Brilliant Blue. The results for both homogenate and ES are shown in Figs. 3.10 and 3.11. These gels also show a number of stage specific molecules which are not apparent in the protease gels. It is interesting to note that the bulk of the protein is present in the 5 molecules between 13 and 20 kDa and the majority of these molecules seem to be conserved, in both ES and homogenates, throughout the development of the parasite. It is also interesting to see that molecules at 19 kDa and 17 kDa seemed to disappear as the parasite aged, being virtually absent by day 240 p.i. The stage specific expression of proteins in the homogenates and ES of the various parasite stages is illustrated in Tables 3.4 and 3.5. Some molecules seem to be of identical molecular weight and stage specific expression as those seen in the protease gels, however, no conclusions could be drawn as the protease gels were run under non-reducing conditions and the protein gels were run under reducing conditions.

Figure 3.10 Protein Gels of Parasite Homogenates

Samples of 100ug of PBS extracted homogenates from various stages of the parasite were run under reducing conditions on a 5-20% SDS-PAGE gel. The gels were stained with Coomassie Brilliant Blue. The molecular weight standards are shown on the left hand side.

(A)	Lane	1	L3	(B)	Lane 1	Day 9
	"	2	Day 1		" 2	Day 10
	"	3	Day 2		" 3	Day 120
	"	4	Day 3		" 4	Day 170
	"	5	Day 4		" 5	Day 240
	"	6	Day 5			
	"	7	Day 6			
	"	8	Day 7			
	"	9	Day 8			


A
MW
kD
94
67
43
30
20
14
1
2
3
4
5
6
7
8
9

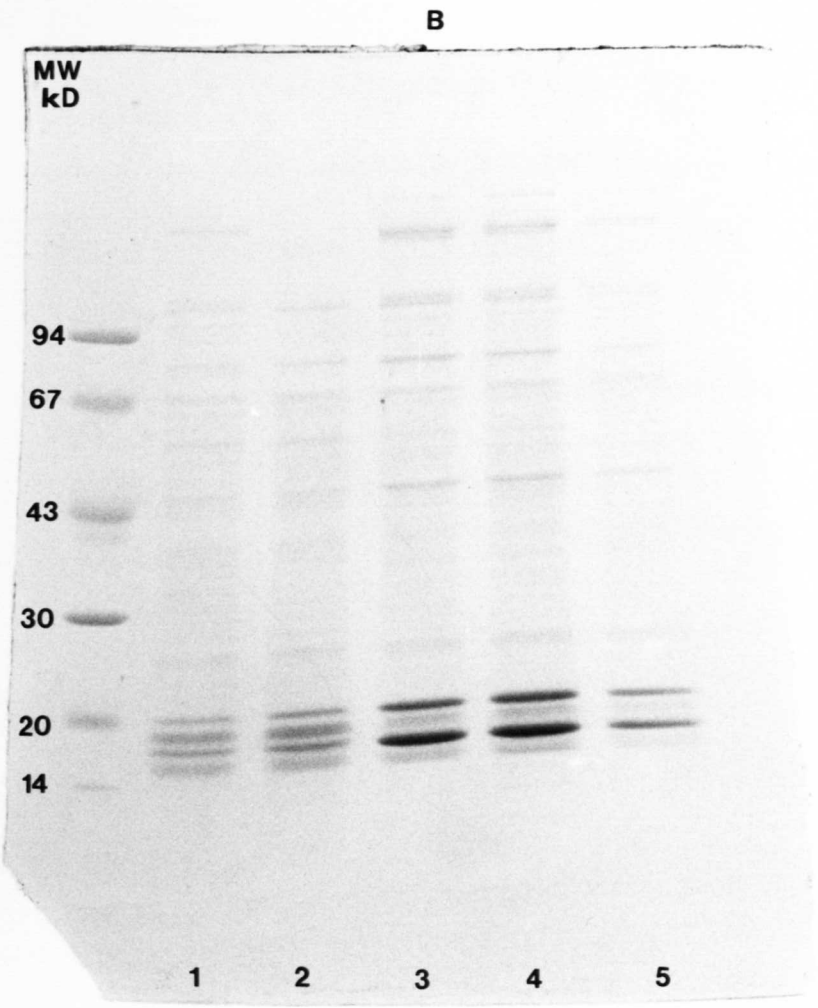

B
MW
kD
94
67
43
30
20
14
1
2
3
4
5

Figure 3.11 Protein Gels of Parasite ES Products

Samples of 100ug of the ES products of various stages of the parasite cultured for 96 hours in the absence of collagen were run under reducing conditions on a 5-20% SDS-PAGE gel. The gels were stained with Coomassie Brilliant Blue. The molecular weight standards are shown on the left hand side.

(A)	Lane 1	Day 1	(B)	Lane 1	Day 10
	" 2	Day 2		" 2	Day 10 Hom.
	" 3	Day 3		" 3	Day 120
	" 4	Day 4		" 4	Day 120 Hom.
	" 5	Day 5		" 5	Day 170 Hom.
	" 6	Day 6			
	" 7	Day 7			
	" 8	Day 8			
	" 9	Day 9			

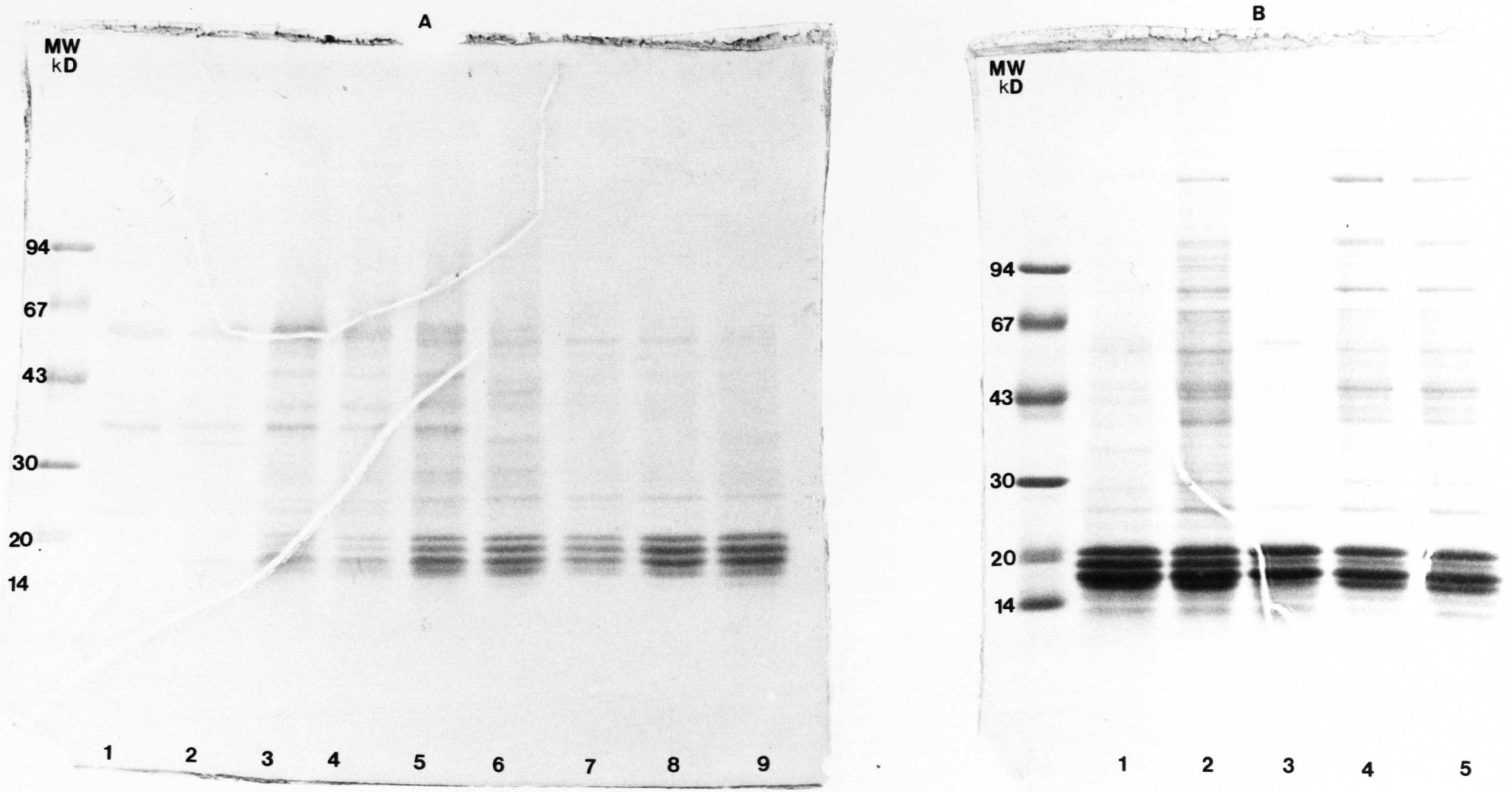

A
MW
kD
94
67
43
30
20
14
1
2
3
4
5
6
7
8
9
B
MW
kD
94
67
43
30
20
14
1
2
3
4
5

Table 3.4 Proteins Present in Parasite Homogenates

The protein molecules present in homogenates of the various stages of the parasites were calculated from Coomassie stained gels and are summarised in the table.

Stage	Molecules Present (kDa)
L3	- - 116 89 - - 47 - 29 - - - 18 - 15 -
Day 1	Bands not visible
Day 2	Bands not visible
Day 3	Bands not visible
Day 4*	- - - 89 80 62 47 41 - - 20 - 18 17 15 13
Day 5	- - - 89 80 62 47 41 - - 20 - 18 17 15 13
Day 6#	- - - 89 80 62 47 41 - - 20 - 18 17 15 13
Day 7	- - 116 89 80 62 47 41 - - 20 - 18 17 15 13
Day 8	- - 116 89 80 62 47 41 - - - - 18 17 15 13
Day 9	- 153 116 89 80 62 47 41 29 25 - 19 18 17 15 13
Day 10	- 153 116 89 80 62 47 41 29 25 - 19 18 17 15 13
Day 120	180 153 116 89 80 62 47 41 29 25 - - 18 - 15 13
Day 170	180 153 116 89 80 62 47 41 27 24 - - 18 - 15 13
Day 240	180 153 116 89 80 62 47 41 27 24 - - 18 - 15 13

N.B.* Moult from L3 to L4
Moult from L4 to adult

Table 3.5 Proteins Present in Parasite ES Products

The protein molecules present in the ES products of the various stages of the parasites were calculated from Coomassie stained gels and are summarised in the table.

Stage	Molecules Present (kDa)															
Day 1	-	-	-	-	-	62	-	-	35	33	-	-	-	19	-	-	15
Day 2	-	-	-	-	-	62	-	-	35	33	-	-	-	19	-	-	15
Day 3	-	-	-	-	-	62	47	41	35	-	-	25	20	19	18	17	15
Day 4*	-	-	-	-	-	62	47	41	35	-	-	25	20	19	18	17	15
Day 5	-	109	-	-	-	62	47	41	35	-	29	25	20	19	18	17	15
Day 6#	-	109	-	-	-	62	47	41	-	-	29	25	20	19	18	17	15
Day 7	-	109	-	-	-	62	47	41	-	-	29	25	20	19	18	17	15
Day 8	-	109	-	-	-	62	47	41	-	-	29	25	20	19	18	17	15
Day 9	-	109	-	-	-	62	47	41	-	-	29	25	20	19	18	17	15
Day 10	146	-	82	73	-	62	47	41	-	-	29	25	20	19	18	17	15
Day 120	146	109	82	73	63	62	47	41	-	-	29	25	20	19	18	17	15

N.B.* Moult from L3 to L4
\# Moult from L4 to adult

3.2.6 Cross Reacting Antigens

Adult ES was separated on a 5-20% SDS-PAGE gel and blotted onto nitrocellulose. In order to assess the stage specificites of antigens, the strips were probed with rabbit anti-day 6 homogenate and rabbit anti-day 4 homogenate. It was seen (Fig. 3.12) that the molecules present in adult ES had more similarity with those present in day 6 worms than in day 4 worms. Molecules at 95, 78, 57, 30, 27, 20, 18 and 17 kDa were seen by the anti-day 6 but only molecules at 57, 30, 20 and 17 kDa were seen by the anti-day 4 serum. No molecules were recognised by a normal serum control. The results are summarised in Table 3.6.

Figure 3.12 Immunoblot of Adult ES Probed with Various Rabbit Anti-Larval *H. polygyrus* Sera

1 mg of day 10 ES was run under reducing conditions on a 5-20% SDS-PAGE gel. Following electrophoretic transfer to a sheet of nitrocellulose, the resulting immunoblot was cut into 5mm strips, probed with rabbit anti-sera that had been raised against day 4 and day 6 larval *H. polygyrus* and developed with HRP-Protein A. Values for molecular weights are shown on the left.

Lane 1 Rabbit anti-day 6
Lane 2 Rabbit anti-day 4

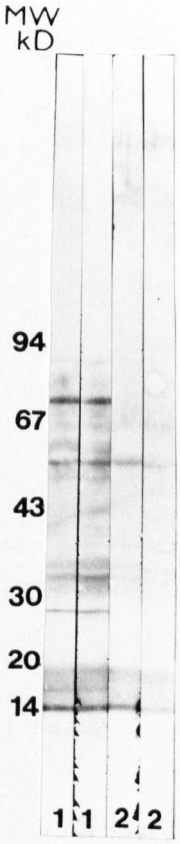

MW
kD
94
67
43
30
20
14
1
1
2
2

Table 3.6 Molecules common to day 4, day 6 and adult worms.

Molecules common to day 6 and adult (kDa)							
95	78	57	30	27	20	18	17
Molecules common to day 4 and adult (kDa)							
-	-	57	30	-	20	-	17

3.3 DISCUSSION

3.3.1 Parasite Growth

It was apparent that there was slight decrease in the length of the parasites from the infective L3 to day 1 p.i. This would have been consistent with the loss of the parasite sheath, the partially shed second stage cuticle. The parasite may have also decreased in size due to it using up its energy supplies in order to exsheath and invade the host mucosa. Up until day 3 the worm did not grow significantly, however, between days 3 and 4, the parasite appeared to grow by up to 3 times its size. This was probably because the parasite had moulted from L3 to L4 at this time and so provided the stimuli for rapid growth. This was also the time when sexual differentiation started to occur. There was a gradual increase in the size of the parasite until and after day 10. Overall, this indicated that the cuticle of the parasite must be a flexible, dynamic structure, unlike an insect cuticle, as growth was able to take place between moults and sloughing of the cuticle was not required for an expansion in size. The female parasites grew much more rapidly than the male worms and attained a greater adult size, being about 3 times the size of the male worms. The male parasites did not appear to grow at all after day 6 p.i., whilst the female parasites continued growing well after they had moulted from L4 to adults. The reason for this is probably because the bulk of the female body is concerned with egg production, with maximum poduction occurring at day 30 p.i.

The similarity between the size of the parasites pre-, and 96 hours post-culture was interesting because it indicated that the parasite was not growing and developing as it would have done in the host. For instance, a day 3 post-ES parasite would be thought to resemble a day 7 worm after the period in culture it would be 4 days older. This is clearly not the case in this culture system. The lack of development is probably due to the absence of the relevant stimuli to promote the

development of the parasite. This has its advantages and its disadvantages, firstly, it means that antigens are produced from the parasite for a longer time and so more could be collected, but secondly, it is not certain if those molecules produced are representative of those produced by the parasite during development in the host. However, preliminary experiments analysisng ES molecules produced by day 6, 10, 20 and 40 after 24, 48, 72, 96 and 120 hours in culture indicated that although the amount of protein produced per day decreased the protein profile did not alter. In order to assess the effect of *in vitro* culture on the development further, it would be necessary to implant various stages of the parasite after different time points after culture and investigate if normal development resumes.

3.3.2 Parasite Culture

In an attempt to try to mimic the host-parasite interactions, a diphasic medium consisting of an insoluble collagen raft and a liquid medium was used to culture the parasite. This could allow the parasites to fix themselves to the collagen raft in a way which may resemble their attachment to the host intestine. However, the results of this experiment indicated that that this did not provide a satisfactory environment for the development of the parasite as illustrated by the protein and enzyme production of the various stages of the parasite. In fact, the parasite seems 'happier' in the normal culture conditions. This could be due to collagen leached from the matrix 'blocking' the parasite's oesphagous and preventing them from feeding and/or secreting enzymes. It is also possible that the parasite is happier in this medium and that the reduced protein and enzyme secretion is due to the worm being less stressed and so not stimulated to produce these products. It has been suggested that *Nippostrongylus brasiliensis* produces increased amounts of AChE in the host when it is subjected to an immune response from the host and when it is implanted into a naive host the production of the

enzyme declined (Ogilvie, 1972). However, it was also demonstrated (Burt and Ogilvie, 1975) that when lactalbumin was incorporated into the culture medium there was a 5 fold increase in the AChE production and Pritchard, Leggett, McKean and Rogan (unpublished observations) showed that when *N. americanus* was cultured on collagen rafts there was a 2 fold increase in the AChE production of the parasite.

3.3.3 Stage Specific Acetylcholinesterase Production

The results from the stage specific release of AChE and PChE using the monophasic culture system were very interesting. AChE, is a true acetylcholinesterase, whose only substrate is acetylcholine, whilst pseudocholinesterase is a non-specific esterase with a wide variety of substrates. It was noted that there was a stage specific release of AChE into the ES products of the parasite, whilst that of PChE did not seem to vary markedly. The maximal production of AChE was between day 4 and 7 p.i., with the adult parasite producing very little of the enzyme. This time of maximum production corresponds with time when the parasite is in the fourth larval stage (L4). The moult from L3 to L4 occurs between 90-96 hours p.i. and that of L4 to adult occurs 144-166 hours p.i. (see life cycle in Chapter 1). In the homogenate, which probably reflects the endogenous somatic level of the enzyme in the parasite such as that needed for neurotransmission, it can be seen that again the maximum AChE levels were present between 4 and 7 days p.i., again, reflecting the period when the worm is in the fourth larval stage. The levels declined quite rapidly to very small amounts when the parasite was an adult. In contrast to that seen in ES, the PChE levels also showed some stage specific production, with the amounts present from L3 to day 3 p.i. comparable to the AChE levels. This suggested that this form of esterase may be important in the early stages of the infection but not to the mature stages. The levels steadily declined and were comparable to the low AChE levels present in the adult worm. It was not clear from this experiment whether

the AChE seen in the ES was merely a result of that leaching out from the parasites or from dead or dying worms or if it was actively secreted into the medium.

There have been a number of reports of stage specific production of AChE by nematode parasites. Sanderson and Ogilvie (1971) showed a 15 fold increase in activity in 17 days from the egg to the adult of *N. brasiliensis* and a 2 fold increase thereafter. The activity of adult *Turbatrix aceti* was twice that of the larval stages but this was followed by a general decline as the parasite aged (Erlanger and Gershon, 1970). Adult and L4 *N. americanus* secrete AChE but the larvae do not (Burt and Ogilvie, 1975). However, the reverse trend has also been demonstrated to be present, with larval parasites secreting more than the adult stage. The microfilariae of *Brugia malayi* secrete 4 times the amount of adults, *Haemonchus contortus* L3 secrete greater amounts than the adult worm (Lee and Hodsden, 1963; Hart and Lee, 1966) as do the L3 of *Trichostrongylus colubriformis* and *Oesophogastomum radiatum* (Ogilvie, Rothwell, Bremner, Schnitzerling, Nolan and Keith, 1973). Different patterns of secretion have also been observed between the sexes of the same parasite; *B. malayi*, *Stephaurus dentatus* and *H. polygyrus* males secrete more than females (Rathaur, Robertson, Selkirk and Maizels, 1987; Rhoads, 1981; Sharpe and Lee, 1981; Mallet, 1989) whilst *O. venulosum* males secrete less than the females (Bremner, Ogilvie, Keith and Berrie, 1973) and in the case of *N. brasiliensis* the two sexes secrete equal amounts (Sanderson, 1972).

This stage specific secretion of the enzyme is not thought to be merely due to increased neuro-muscular function as most cells are thought to be fully differentiated after hatching (Gershon, 1970). It has also been demonstrated that adult *N. brasiliensis* release 10% of their AChE *in vitro* in 1 hour (Sanderson and Ogilvie, 1971) and in 24 hours, 4 times the initial level of AChE was released (Burt and Ogilvie, 1975). *S. dentatus* was reported to release 45% of the total amount of AChE in 20 hours (Rhoads, 1981). It has been demonstrated previously that *H. polygyrus* releases very little AChE

(Sanderson, 1972), with only 1% of the total worm enzyme activity being released per hour, which was about one third of that of *N. brasiliensis*. It was also proposed that the low amounts produced by *H. polygyrus* were because the enzyme present was primarily associated with the neuro-muscular system (Sharpe and Lee, 1981). Although AChE is associated with neuro-muscular transmission and is associated with the nervous system, namely the amphids, phasmids, cephalic papillae and nerve ring (McLaren, Burt and Ogilvie, 1974; Omar and Kuhlow, 1977) it is also found in the secretory glands of a number of nematodes such as *N. brasiliensis* (Lee, 1970), *N. americanus* (Mclaren, Burt and Ogilvie, 1974), *T. colubriformis* and *O. radiatum* (Ogilvie et al, 1973).

In other systems AChE is usually associated with nerve transmission and neuro-muscular junctions, but it is also found in erythrocytes, platelets, snake venom and the blood of *Helix* spp. suggesting that it has more than one role (Ogilvie et al, 1973). *T. colubriformis, Turbatrix aceti, O. radiatum, O. venulosum* and *N. brasiliensis* all secrete soluble AChE in large amounts not associated with neuro-transmission, whereas, *H. contortus, Ascaris lumbricoides, H. placei, Trichinella spiralis, Trichuris muris* and *H. polygyrus* all secrete the enzyme in small amounts and the majority was found to be associated with the particulate fraction i.e. nervous tissue. There are no free-living or plant dwelling nematodes which secrete large amounts of AChE (Lee, 1964; Rhode, 1960; Bird, 1966; Erlanger and Gershon, 1979). Thus, it emerges that AChE must be playing some role other than neuro-transmission in animal parasitic nematodes.

Previous reports of low levels of AChE found in *H. polygyrus* could have been because other authors (Ogilvie et al, 1973; Sanderson, 1972) used the adult rather than the larval stage of the parasite. It has been demonstrated in this study that the larvae produced significantly larger amounts of the enzyme than did the adults.

At first it was proposed that the enzyme was acting as a 'biological holdfast' by causing local paralysis of the host

gut and thus inhibiting the peristaltic movements of the intestine and so delaying the expulsion of the parasite (Lee, 1970; Ogilvie and Jones, 1971). Indeed it has been shown that passage of ingesta through the gut was slowed in *N. brasiliensis* infected rats (Symons, 1966) and in sheep with *O. columbianum* (Gershon, 1970).

More lately it has been hypothesised that AChE may contribute to parasite survival by interfering with the process of mucus secretion by intestinal goblet cells (Philipp, 1984). Mucus has long been implicated with the expulsion of gastrointestinal nematodes such as *T. spiralis* in immune rats (Lee and Ogilvie, 1980) and *N. brasiliensis* where goblet cell hyperplasia develops during the infection (Miller and Nawa, 1979). Parasympathetic control of gastrointestinal mucus secretion has been demonstrated both *in vivo* and *in vitro* (Specian and Neutra, 1980; 1982). Micromolar amounts of acetylcholine can induce massive mucus secretion from rabbit intestinal crypt-goblet cells (Specian and Neutra, 1980). Therefore, it seems reasonable that AChE produced by nematodes in the gut could counteract the effects of acetylcholine and thus delay worm expulsion.

AChE is also thought to be capable of regulating the hosts immune and inflammatory responses through messenger and membrane-receptor modulation (Rhoads, 1984). Acetylcholine and related agonists also enhance and promote the release of histamine and leukotrienes from mast cells (Kaliner, Orange and Austen, 1972; Ignarro and George, 1974), promote the ability of cytotoxic lymphocytes to kill target cells (Strom, Stykowski, Carpenter and Merrill, 1974). They have also been implicated in numerous effects on polymorphonuclear cell functions such as lysosomal enzyme secretion, phagocytosis and antibody dependent cellular cytotoxicity (Ignarro and George, 1974; Kaliner and Austen, 1974). Acetylcholine has also been shown to raise intracellular cGMP levels and thus T cells are no longer dependent on IL-2 for activation and subsequently for gamma interferon production (Johnson, Archer and Torres, 1982) and thus immune activity may be directly depressed by the enzyme.

Many other T cell functions could also be affected as they too bear an acetylcholine receptor (Strom, Stykowski, Carpenter and Merrill, 1974; Fuchs, Schmidt-Hopfeld, Tridente and Tarrab-Hazdai, 1980). Mouse spleen PFC can also be suppressed by AChE to both T-independent and T-dependent antigens (Johnson, Smith, Torres and Blalock, 1982).

Therefore, it seems that if the host is to expel the parasite, inactivating the AChE produced could not only dislodge the parasite but elevate the immune response of the host to the parasite. A number of authors have reported the presence of antibodies to parasite AChE, for example, AChE is recognised by serum from *B. malayi* and *Wuchereria bancrofti* infections (Rathaur, Robertson, Selkirk and Maizels, 1987), *N. brasiliensis* (Jones and Ogilvie, 1972), *O. radiatum* (Bremner, Ogilvie, Keith and Berrie, 1973), *S. dendatus* (Massoulie and Bon, 1982), *N. americanus* (Ogilvie et al, 1978) and *T. colubriformis* (Rothwell and Merritt, 1974). However, it is unclear whether these antibody responses have any effect on the establishment or survival of the parasite as it has been demonstrated that although antibodies are produced they may not inactivate the enzyme. This suggests that they may bind without inhibiting the activity, alternatively they may even enhance the activity by binding the enzyme to cell surfaces through the interaction of the Fc portion of the antibody (Williams, 1969). It has also been shown that IgG_1 anti-*T. colubriformis* AChE antibodies could prevent the penetration of AChE to sites of acetylcholine release and thus indirectly inhibit its effects (Rothwell and Merritt, 1974), However, attempts to immunise animals with purified AChE have been largely unsuccessful mainly due to the lack of material (Rothwell and Merritt, 1975). It may also possibly be because it has been shown that if antibodies to AChE are injected into naive rats which are then implanted with *N. brasiliensis*, the parasites increase their production of AChE. If cortisone is injected into immune rats the AChE production of the parasites decreases (Ogilvie, 1972). Pritchard, Leggett, McKean and Rogan (unpublished observations) demonstrated that IgG

preparations of an anti-serum raised against purified *N. americanus* AChE could inhibit AChE activity *in vitro* and that when parasites were incubated with this sera, their ability to to secrete AChE was inhibited. Jones, Edwards and Ogilvie (1972) reported that there was no correlation between antibodies to the parasite and host protection. However, it was found (Jones and Ogilvie, 1970) that the proportions of iso-enzymes altered in response to an immune reaction. Iso-enzymes B and C increased in both damaged and immune adapted worms, but isoenzyme A decreased in damaged worms and increased dramatically in adapted worms and so they proposed that this was the parasite protective enzyme.

A number of attempts have been made to isolate the AChE molecule in a number of parasite species. There are a large number of different enzymes and they are not conserved between parasite species. It has been reported to exist as a single molecule (Rothwell, Ogilvie and Love, 1973; Rhoads, 1981) and as a multi-molecular form (Edwards, Burt and Ogilvie, 1971). It is known to be both soluble and membrane-bound (Yeates and Ogilvie, 1976; McLaren, Burt and Ogilvie, 1974). The molecular weight is known to vary between 60 and 100 kDa (Hogarth-Scott et al, 1973; Rhoads, 1981; Watts and Atkins, 1981) and *N. americanus* AChE is reported to be 400 kDa (Yeates and Ogilvie, 1976). However, Pritchard, Leggett, McKean and Rogan (unpublished observations) reported it as being a multi-molecular form of 32, 60, 80, 140 and 220 kDa.

In *H. polygyrus* ES, the anti-sera which detected 5 molecules in *N. americanus* ES only recognised one molecule of 30.5 kDa. It is possible the the molecule seen here at 30.5 kDa may be the 32 kDa molecule seen in the *N. americanus* preparation. The reason why the other molecules may not have been recognised could be because other molecules did not bear the same epitopes and so did not cross-react with *N. americanus* AChE, or that it exists as a single molecule which may associate in sub-units.

3.3.4 Stage Specific Protease Production

Parasite proteases have been extensively studied for a number of years. They are enzymes which catalyse the hydrolysis of peptide bonds and thus disrupt proteins of either parasite or host origin. A large number of functions in various parasite species have been attributed to this group of enzymes, these include, invasion of host tissues, digestion of host proteins, evasion of the hosts immune response and prevention of blood coagulation (McKerrow, 1989). Proteases at different life cycle stages are also thought to trigger or contribute to morphological changes and allow metabolism within the host (McKerrow and Doehnoff, 1988).

In order to quantitatively assess the changes occurring, an assay system was devised. This was essentially as described by Robertson, Kwan Lim and Maizels (1988). An ideal protease assay system should be highly sensitive, require short incubation times, have low background readings and the substrate should resemble the native substrate (Sevier, 1976). This assay fulfilled all of these requirements adequately and so was used to test a range of parasite ES and homogenates. This simple, sensitive assay made use of the substrate casein, which can be digested by a broad range of enzymes and allowed a large number of samples to be screened at once. Although the assay was essentially that as described by Robertson, Kwan Lim and Maizels (1988), the substrate was not dried onto the plates at 37^{o}C for safety reasons. As a result, the counts that bound per well were fairly low (953.5 $\pm$ 49.4) compared with 10,000 counts that were loaded onto each well. Nevertheless, this was still sufficient to detect a 5% release of substrate from the plate. As can be seen from the results of the standard tryptic curve, there was little error, and the assay was sensitive to as little as 1 ng of trypsin. It was noted that there was high percentage release between days 1 and 4 which declined thereafter, with the amount in the homogenates being comparable to the amounts in ES. When this was converted to trypsin activity, due to the logarithmic nature of the standard curve,

the profile looked quite different. The day 1 preparation had a very high activity and this rapidly declined to about one twentieth for days 2-4. By day 5 the amount of protease activity detectable was negligible compared with day 1. Interestingly, L3 showed very little activity. It seems that there is a large amount of protease secreted for just one day and that after this the enzyme may not have been so important. It was surprising that there was not more activity observable in the L3 preparation as they have been demonstrated to contain large amounts of leucine aminopeptidase (LAP) which is involved in the exsheathing of the parasite when it enters the host. However, this may be because its production has not yet been stimulated as it has not encountered the relevant stimuli. The large amounts of enzymes present at day 1 could be due to two factors. Firstly, it could be large amounts of the enzyme required for the infective larvae to exsheath still being produced. These parasites were collected from the host 24 hours p.i. at which time it has been reported that some of the parasites are still in the process of losing their sheaths (Bryant, 1973), although most of the worms have lost their sheaths within 1 hour (Ey, 1988b). The second factor which could be responsible for the large amounts of proteolytic enzymes produced could be that the enzymes are required for the parasite to invade the intestinal mucosa and muscle layer where it develops into the fourth larval stage and adult before it re-emerges into the intestinal lumen. The activity on days 2 and 3 could be due to enzymes needed for the breakdown of host tissue necessary for parasite feeding. Those enzymes present on day 4 could be associated with the developmental proteins required for the moult from L3 to L4. Although, there are enzymes present from day 5 onwards, they are about one hundredth the level on day 1 and so are not well represented graphically, but can be seen on the table. The enzymes that are present are probably again involved in feeding, development and emergence of the adult parasite into the intestinal lumen. The casein substrate gels confirm the findings of the assay. Activity appears to be much stronger in the first few days of

the infection and was comparable in both the ES and homogenates. The change in the pattern of protease activity indicated that more than one enzyme was present at any stage in the parasite life cycle, although as the parasite matured and aged, the numbers of proteases present declined. This was probably because the numbers of molecules required were less when the parasite was in the lumen as it did not have to digest the host tissue. The enzymes that were present were probably necessary for feeding and possibly evasion of the host immune response.

The results of this study are in contrast to the work reported by Monroy, Cayzer, Adams and Dobson (1989) where they suggested that activity in extracts of L3 and L4 was negligible against collagen, casein or fibrinogen. Even in adult ES they only found activity at 48 kDa against casein and fibrinogen, although activity was also found at 105 and 200 kDa in gelatin substrate gels. These are possibly equivalent to the 42, 102 and 116 kDa molecules seen in this study. The fact that protease activity in larval preparations has not previously been demonstrated could be the result of the poor yield of these parasite stages that the authors have reported (Adams, East, Monroy, Washington and Dobson, 1987). The methodology used in the current study may also be more sensitive as a lower concentration of casein was used in the gels, and so less enzyme activity would be required to produce detectable digestion. Thus, it was seen that this system, utilising casein, revealed more molecules than has been previously demonstrated for *H. polygyrus*.

Knox and Kennedy (1988) demonstrated that there was a stage specific protease production in *Ascaris suum*. L2, L3 and L4 ES exhibited a variety of proteinolytic activities with multiple pH optima, substrate specificities and inhibition profiles. As in this study, there were different somatic (homogenate) and secreted enzymes, and L3/L4, equivalent to days 1-4 in this study, released markedly more enzyme than adults or L2.

The infective larvae of *T. canis* were shown (Robertson, Bianco, McKerrow and Maizels, 1989) to have very high levels of proteolytic enzymes. The material released from a R22 matrix by *T. canis* larval ES consisted of 16% collagen, 21% elastin and 66% trypsin-labile glycoproteins. ES of the larvae of *Strongyloides* (Rege and Dresden, 1987), *Brugia* spp. (Petralanda Yarzabaha and Piessens, 1986), hookworm (Hotez, LeTrang, McKerrow and Cerami, 1985), *D. immitis* (Tamashiro, Rao and Scott, 1987) and *Onchocerca* spp. (Lackey, et al, 1989) have all been shown to contain proteolytic activity.

The role of this great variety of proteases is not fully understood, but a number of functions have been elucidated for a few. Several skin invasive larval nematodes have been demonstrated to have elastinolytic proteases (Rege and Dresden, 1987; Hotez et al, 1985). Other parasites such as *Anisakis simplex* (Sakanari and McKerrow, 1988) and *Entamoeba histolytica* (Keene, Petitt, Allen and McKerrow, 1986) have a number of collagenases but no elastases. It had been proposed that this is because they do not migrate through the elastin-rich skin and lungs, but do invade the collagen-rich intestine wall (McKerrow and Doenhoeff, 1988). Many filarial L3 secrete proteases active against gelatin, collagen and elastin, again to facilitate migration through the skin (Lackey et al, 1989) and *Ascaris suum* larvae which invade both the bowel wall and lung interstitium appear to have a broad spectrum of proteases (Knox and Kennedy, 1988). Many blood feeding parasites also secrete haemoglobinases e.g. schistosomes (Chagell and Dresden, 1986; 1987), hookworms (Hotez and Cerami, 1983) and *D. immitis* (Tamashiro, Rao and Scott, 1987). Fibrinolytic enzymes are also actively secreted by a number of blood feeding parasites, presumably to prevent clots from forming whilst feeding, e.g. *Ancylostoma caninum* (Thorsen, 1956a,b; Hotez and Cerami, 1983). Parasite proteases may also be involved in the evasion of host protective immunity through the proteolytic degradation of antibodies. Immunoglobulin bound to the surface of *S. mansoni* schistosomula and free IgG are hydrolysed by parasite derived proteases (Auriault, Ouiassi, Torpier, Easier and Capron,

1981). The peptides produced inhibit macrophage stimulation in addition to the removal of immunoglobulin Fc from the parasite surface. Such a mechanism has also been proposed as a possible strategy for immune evasion by immature *Fasciola hepatica* via the secretion of a 'fabulating' enzyme which cleaves antibody to release the Fc portion of the binding antibody (Chapman and Mitchell, 1982). *D. immitis* is also known to hydrolyse IgG to produce a 22 kDa molecule (Tamashiro, Rao and Scott, 1987). It has been further demonstrated that a group of bacterial enzymes can specifically cleave IgA (Plaut, 1983). Parasites may also produce enzymes which degrade their own molecules. *S. mansoni* produces two serine proteases which accelerate release of molecules from the schistosome membrane (Marikorski, Arnon and Fischelson, 1988). It is thought that this allows the release of surface proteins with attached complement and thus makes the transforming schistosomula refractory to complement damage.

Thorsen (1956a) demonstrated that a protease produced by *A. caninum* was inhibited by immune dog serum. Hotez and Cerami (1983) also showed that *A. caninium* fibrinolytic enzyme was partially inhibited by immune dog serum. *S. mansoni* haemoglobinase has a marked IgG and IgE response to it (Zerda, 1987) and a thiol protease from *E. histolytica* is recognised by immune serum (Toy et al, 1987). *A. suum* proteases were inhibited by serum antibody from infected hosts (Knox and Kennedy, 1988) and Plaut (1983) demonstrated that secretory IgA contained a population of antibodies capable of binding and inhibiting the enzyme activity of bacterial IgA_1 proteases. Non-antibody host responses to proteases may also occur. For instance, serum contains a range of protease inhibitors which can regulate both host and parasite proteases and it has been shown (Modha, 1988) that contrapsin, a mouse serine protease inhibitor, binds to adult schistosome homogenates.

The major surface protein of *Leishmania major* is a stage specific metalloprotease. It is thought to facilitate the entry of the parasite into macrophages by cleaving C3b to C3bl and binding to the surface receptor (Bordier, 1987). Proteases are thought to be useful proteins for serodiagnosis. Cathepsin

L-like proteases have been found in the serum of *S. mansoni* (Zerda, 1987), *Plasmodium falciparum* and *E. histolytica* infections (Rosenthal, Kim, McKerrow and Leech, 1987; Keene, Petitt, Allen and McKerrow, 1986). Therefore, the isolation of parasite proteases would be useful in not only serodiagnosis but also in immunotherapy and chemotherapy (Ottesen, 1988).

3.3.5 Stage Specific Protein Production

From the Coomassie stained protein gels it was seen that the main protein bands did not appear to correlate with the main areas of protease activity. In fact, the bands which corresponded with the protease bands could not be detected, this could be because enzymes can often be detected in the picogram range (Spanos and Hubscher, 1983). Alternatively, it may have been because the protease gels were run unreduced in order to conserve the enzymic activity (Lacks and Springhorn, 1980), whilst the protein gels were run reduced and boiled. Some enzymes are not B-ME sensitive but in order to conserve as much native activity as possible the samples were not subjected to this treatment. SDS also affects some enzymes but is necessary to run the samples. To minimize the effects of this, the gels were gently washed in Triton X 100 which allows the gradual renaturation of the proteins without damaging them. Reducing conditions were also not applied because if some of the enzymes consisted of a number of different sized sub-units they would not be able to re-associate when separated on the gel (Lacks and Springhorn, 1980).

3.3.6 Cross Reacting Antigens

From these results it was noted that although there were a number of molecules that were common to day 4, day 6 and adult parasites, the numbers of molecules that were recognised by the anti-day 4 serum was minimal. This then suggests that the development of the parasite results in the expression of a number of stage specific proteins and that there are an

increasing number of common antigens as the parasite matures. This implies that an immune response against the larval stage of the parasite may not be completely effective at damaging the adult stage.

Other parasite enzymes may also be important in the host parasite relationship as the action of lipases produced by parasites can be inhibited by immune serum but not by naive serum (Thorsen, 1953). The role of parasite anti-oxidants has also been proposed to be a mechanism by which the parasites are able to survive in a potentially damaging host immune response. Kazura and Meshnick (1984) found a good correlation between the susceptibility of the developmental stages of *T. spiralis* to the immune system and the levels of enzymes protective against free-radical damage. *H. polygyrus* is more resistant to free-radical damage than is *N. brasiliensis* which is expelled rapidly from the host during a primary infection. This susceptibility was related to the levels of protective enzymes; *H. polygyrus* had double the levels of superoxide dismutase and 3-4 times the levels of catalase contained in *N. brasiliensis*. There were also high levels of glutathione reductase which provides high levels of reduced glutathione, a free-radical scavenger (Smith and Bryant, 1986; 1989).

In summary, it can be seen that *H. polygyrus* releases a large number of enzymes and in a stage specific manner. It was noted that both AChE and protease enzymes were released in greater quantities in the larval stages than as an adult, hence, it would appear that an immune response to these enzymes could retard development and/or kill the larval stages of the parasite more efficiently than the adult stages. It is well known that immunity to *H. polygyrus* is stage specific and directed against the larvae, with the adults able not only to survive but to suppress the response both to it and to heterologous antigens (Behnke and Robinson, 1985; Behnke and Wakelin, 1977; Enriquez, Cypess and Wassom, 1988; Ey, 1988a,b; Goven and De Buysscher, 1980; Hagan, Behnke and Parish, 1981; Hosier, Sackman and Idell, 1974; Jones and Rubin, 1974; Mitchell and Munoz, 1983; van Zandt, 1973). Accordingly, it

seems possible that the adult parasite is able to avoid attack both through the presence of a different set of antigens and through the possible release of immunomodulatory factors.

It appears that *H. polygyrus* firstly releases proteases in order to exsheath and invade the host mucosa, and then secondly, feeding enzymes and/or enzymes which can combat the host immune response. Whilst the parasite is in the fourth larval stage it is in intimate contact with the host mucosa and thus, the immune response and for this reason it would need to release larger amounts of both proteases and AChE in order to neutralise any responses to it and so facilitate its survival.

CHAPTER FOUR

STAGE SPECIFIC EXPRESSION OF SURFACE PROTEINS OF *HELIGMOSOMOIDES POLYGYRUS*

4.1 INTRODUCTION

Radio-iodination is a technique widely used in order to study the molecules present on the surface of a variety of organisms including, viruses, bacteria, protozoa, trematodes and nematodes. It is known that *H. polygyrus* produces stage specific proteins both in ES and on its cuticle (Pritchard, Maizels, Behnke and Appleby, 1984; Pritchard and Carr, 1987; Adams, East, Monroy, Washington and Dobson, 1987). It is also thought that immune responses are directed at larval proteins as larvae have been demonstrated to be immunogenic whilst adult parasites are able to both avoid and suppress the host immune response directed against them (Ali and Behnke, 1983; 1984; Behnke, Ali and Pritchard, 1983; Cayzer and Dobson, 1983; Colwell and Wescott, 1973; Crawford, Behnke and Pritchard, 1989; Dehlawi and Wakelin, 1988; Jenkins and Behnke, 1977; Losson, Lloyd and Soulsby, 1985; Monroy, Dobson and Adams, 1989; Price and Turner, 1983a,b,c; 1987; Pritchard, Ali and Behnke, 1984; Shimp, Crandall and Crandall, 1975).

Accordingly, this study was aimed to elucidate stage specific proteins on the surface of this parasite which may contribute to the immune response and destruction of the larval stages of the parasite and the survival of the adult.

The nematode cuticle consists of three basic layers, the cortical, matrix and basal layers. The hypodermis which lies beneath the cuticle is thought to be responsible for the generation of cuticular proteins which migrate up through the various layers of the cuticle until they have reached their final position. The cortical layer of the cuticle is the outermost layer and is itself, divided into the outer and inner cortex. The outer layer is also covered by a trilamellate layer and a polyanionic glycocalyx, which together form the epicuticular complex (Lee, 1977; Lumsden, 1975; Inglis, 1983; Murrel and Graham, 1983; Himmelhoch and Zuckerman, 1983). Extraction procedures are useful in order to establish the overall composition of structures such as the cuticle or the tegument and their functional involvement in the worms surface

and the host parasite interplay.

Thus, in order to characterise the molecules and their position in the cuticle, the parasites were subjected to a number of extraction procedures, including, preparation of cuticular proteins using PBS, SDS and B-ME, stripping of the glycocalyx using the detergent, CTAB, and analysis of the hydrophobicity of the molecules using the detergent Triton X 114.

4.2 RESULTS

4.2.1 Preliminary Extractions

Firstly, attempts were made to extract cuticular proteins from a number of parasite stages using PBS only. It was seen (Fig. 4.1) that the differences between day 10 and 30 parasites were negligible with only a greater intensity of the 76 kDa molecule apparent in the day 10 preparation. This could have been the result of inconsistent labelling or the loading of different amounts of sample on the gel. It was also seen that the profile from exsheathed L3 was very different from that of the adult parasites, with no apparent conserved molecules. Interestingly, the purified sheaths from the larvae did not label at all and no bands were present on the gel.

Figure 4.1 PBS Extraction of Surface Labelled Parasites

Parasites were radio-iodinated using the Iodo-gen method and homogenised in PBS, the remaining pellet was boiled in reducing sample buffer. 10,000 cpm of each extract were run under reducing conditions on a 5-20% SDS-PAGE gel and the gel autoradiographed. The molecular weight standards are shown on the left hand side.

Lane 1	PBS Extracted	Day 10
" 2	" "	Day 30
" 3	" "	Exsheathed L3
" 4	" "	L3 Sheaths
" 5	Reduced Pellet	Day 10
" 6	" "	Day 30
" 7	" "	Exsheathed L3
" 8	" "	L3 Sheaths

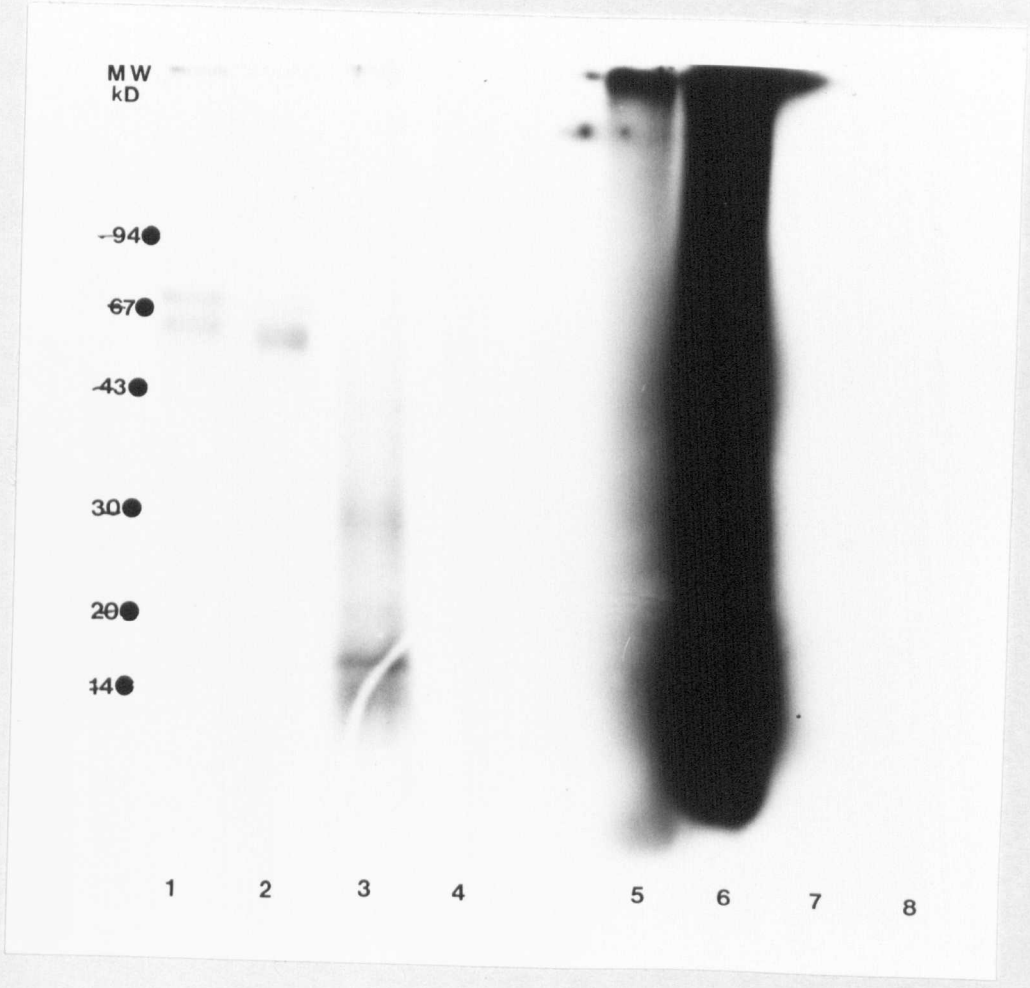

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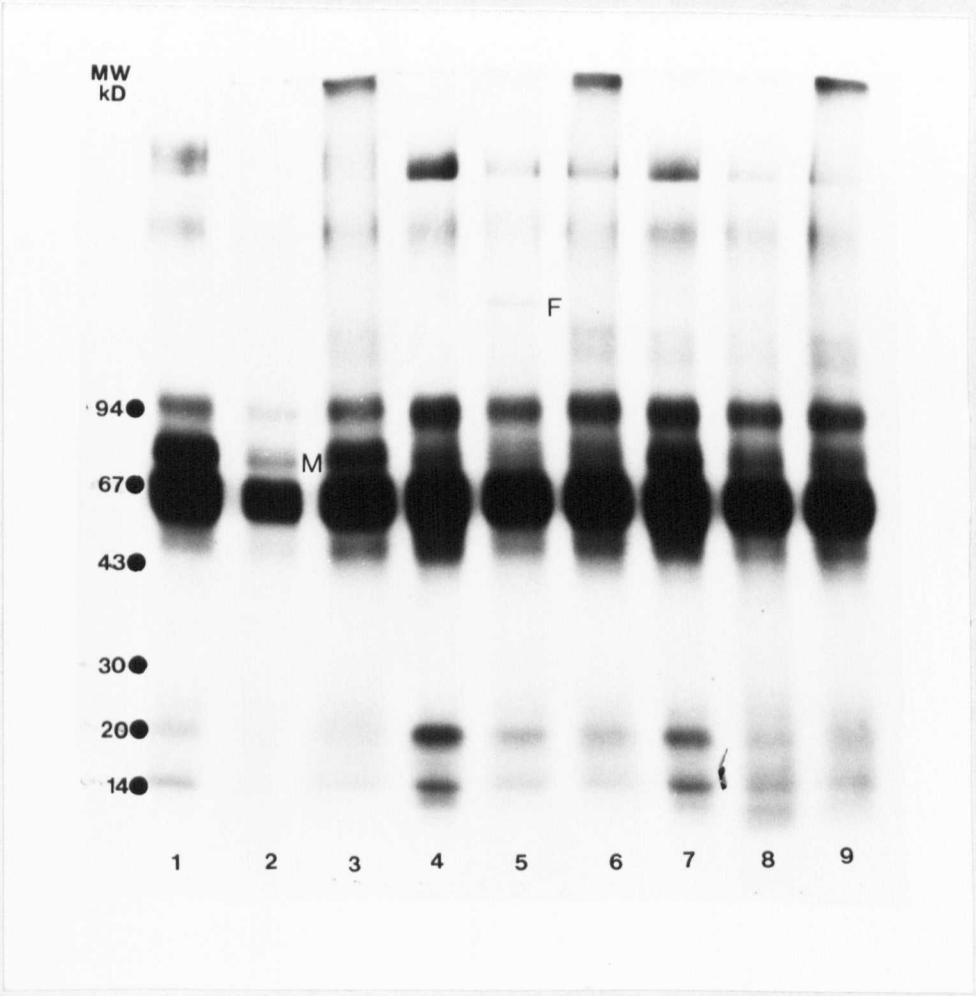
Fig. 4.2 shows the PBS, SDS and B-ME extraction profile of male and female worms. It can be seen that there was a 76 kDa male specific protein and a 145 kDa female specific protein present, with also possibly, 20 and 15 kDa female specific molecules. Bands were seen at these weights in the male preparations, but this could have been due to the presence of contaminating female parasites or their eggs. It was also noted that the 76 kDa molecule was very prominent in the male fractions whilst the 145 kDa molecule was only faintly seen in the female S2 preparations. The molecule at 232 kDa was also possibly female specific; the corresponding molecule in the males appeared to be slightly higher at 237 kDa and was also not labelled as intensely.

Figure 4.2 Surface Protein Profile of Male and Female Parasites

Parasites were radio-iodinated using the Iodo-gen method and then the molecules were extracted firstly using PBS (S1) and then SDS (S2) and finally B-ME (S3). 10,000 cpm of each extract were run under reducing conditions on a 5-20% SDS-PAGE gel and the gel autoradiographed. The molecular weight standards are shown on the left hand side.

Lane 1 Male S1
" 2 Male S2
" 3 Male S3
" 4 Female S1
" 5 Female S2
" 6 Female S3
" 7 Mixed S1
" 8 Mixed S2
" 9 Mixed S3

M = Male specific molecule
F = Female specific molecule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In order to study the properties of the adult cuticle further, the parasites were CTAB stripped before and after labelling (Fig 4.3a). CTAB stripping prior to the labelling procedure not only removes the molecules which are most readily available, but also allows other molecules to be more intensely labelled. The `smeared' pattern on the gel could be attributed to the presence of contaminating detergent (CTAB) interfering with the charge of the parasite proteins, or the presence of labelled lipids (Watt, Burgess and Metcalf, 1979). It was seen that the CTAB stripping removed molecules at 92, 62, 47 and 208 kDa, but did not affect those at 15 or 350 kDa. Not all of the molecules were removed from the surface using the stripping method, but 40% of the label was contained in the stripped supernatant compared with 40% in the remaining 3 fractions, in a normal preparation 75% of the label would be contained in the S1, S2 and S3 fractions. The Triton X 114 extraction of the adult worms (Fig. 4.3b) showed that the majority of the proteins labelled were hydrophilic. The only proteins in the detergent phase were the 92, 62 and 47 kDa molecules and these may have merely represented contaminating proteins. However, the molecules at 145, 208 and 20 kDa were not present which implied that a certain proportion of the proteins may be slightly hydrophilic.

Figure 4.3 Detergent Extraction of Surface Labelled Adult Parasites

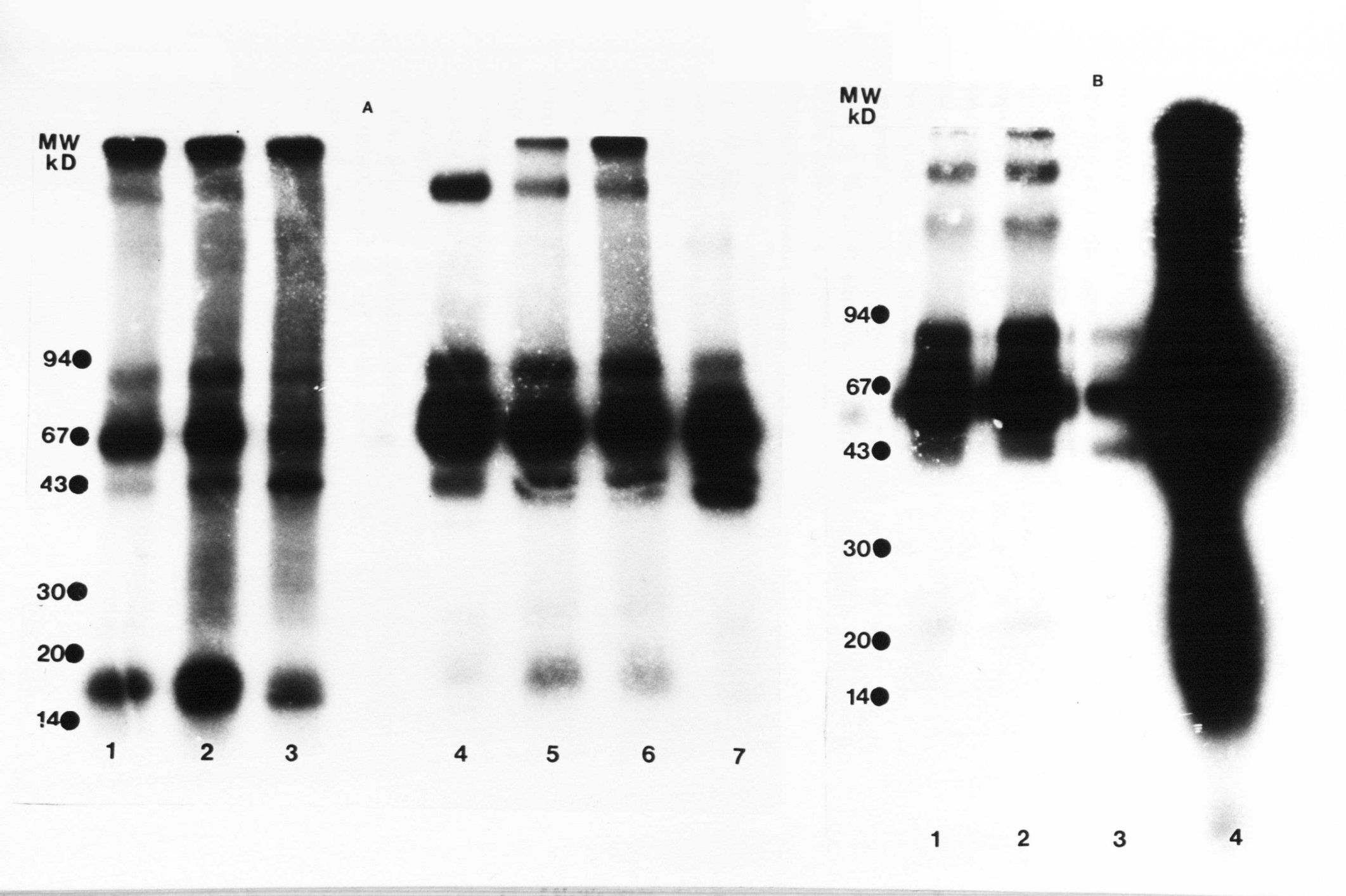
(A) Parasites were radio-iodinated using the Iodo-gen method either before or after they were subjected to surface stripping with the cationic detergent cetyltrimethylammonium bromide (CTAB) and were then extracted with PBS (S1), SDS (S2) and B-ME (S3).

(B) Parasites were labelled as above and then hydrophilic and hydrophobic molecules were extracted using the detergent Triton X 114.

10,000 cpm of each extract were run under reducing conditions on a 5-20% SDS-PAGE gel and the gel autoradiographed. The molecular weight standards are shown on the left hand side.

(A) Lane 1 CTAB Stripped First S1
" 2 " " " S2
" 3 " " " S3
" 4 CTAB Stripped After Supernatant
" 5 " " " S1
" 6 " " " S2
" 7 " " " S3

(B) Lane 1 Triton Extract Aqueous Phase
" 2 " " Sucrose Cushion
" 3 " " Detergent Phase
" 4 " " Reduced Pellet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4.2.2 Cuticle Preparations

Figs. 4.4, 4.5 and 4.6 show cuticle preparations of ensheathed L3 and days 1-10 p.i. Between days 8-10, the molecule at 92 kDa appeared to become more pronounced with age, as did the 20 kDa band. However, the band at 12 kDa seemed to disappear by day 10 but an additional band at 13 kDa had appeared. Bands at 208, 76, 62, 47, 20 and 15 Kda were conserved over this time period. By days 6 and 7 not only were the bands labelled less intensely, but the profile was more restricted. The bands at 208, 76, 62, 47 and 15 kDa were conserved, but those at 92 and 25 kDa were lost. By day 6, the bands at 76 and 47 kDa were lost with those present at 208 and 15 kDa only being faintly visible. In both days a molecule at 17 kDa has appeared and was stronger in the day 6 preparations. By days 4 and 5, the labelled profile was even more restricted, the 62 kDa protein was present in day 5 worms, but was very faint in day 4 parasites. The band at 15 kDa had intensified and bands at 10 kDa had appeared. The label present at the bottom of the gel may be a molecule but it is also possible that it could be free iodine that has passed through the gel. Faint bands were present at 40, 30 and 20 kDa on day 5 but were absent by day 4. The 20 kDa band could have been the same molecule which is present in day 6 parasites. Day 3 worms show an even more restricted pattern of molecules with few of those proteins present in the day 10 parasite apparent in this stage of the worm. The only visible molecules were present at 10 and 12 kDa, with a 32 kDa being faintly visible in the S2 fraction. The molecules at 62 and 15 kDa were completely absent in this stage of the parasite. When days 1 and 2 and ensheathed L3 were labelled, again the profile was very limited, and a very `smeared' preparation was produced. Bands were present in the L3 at 7 and 14 kDa which may have been the same as those molecules located at 10 and 12 kDa in the days 1 and 2. In the L3 preparation bands were also faintly visible at 115, 74, 48 and 40 kDa with protein only being present in the S2 and S3 fractions.

Figure 4.4 Surface Protein Profile of Parasites Aged Between Days 6-10

Parasites were radio-iodinated using the Iodo-gen method and then the molecules were extracted firstly using PBS (S1) and then SDS (S2) and finally B-ME (S3). 10,000 cpm of each extract were run under reducing conditions on a 5-20% SDS-PAGE gel and the gel autoradiographed. The molecular weight standards are shown on the left hand side.

(A)	Lane 1	Day 10 S1	(B)	Lane 1	Day 7 S1
	" 2	Day 10 S2		" 2	Day 7 S2
	" 3	Day 10 S3		" 3	Day 7 S3
	" 4	Day 9 S1		" 4	Day 6 S1
	" 5	Day 9 S2		" 5	Day 6 S2
	" 6	Day 9 S3		" 6	Day 6 S3
	" 7	Day 8 S1			
	" 8	Day 8 S2			
	" 9	Day 8 S3			

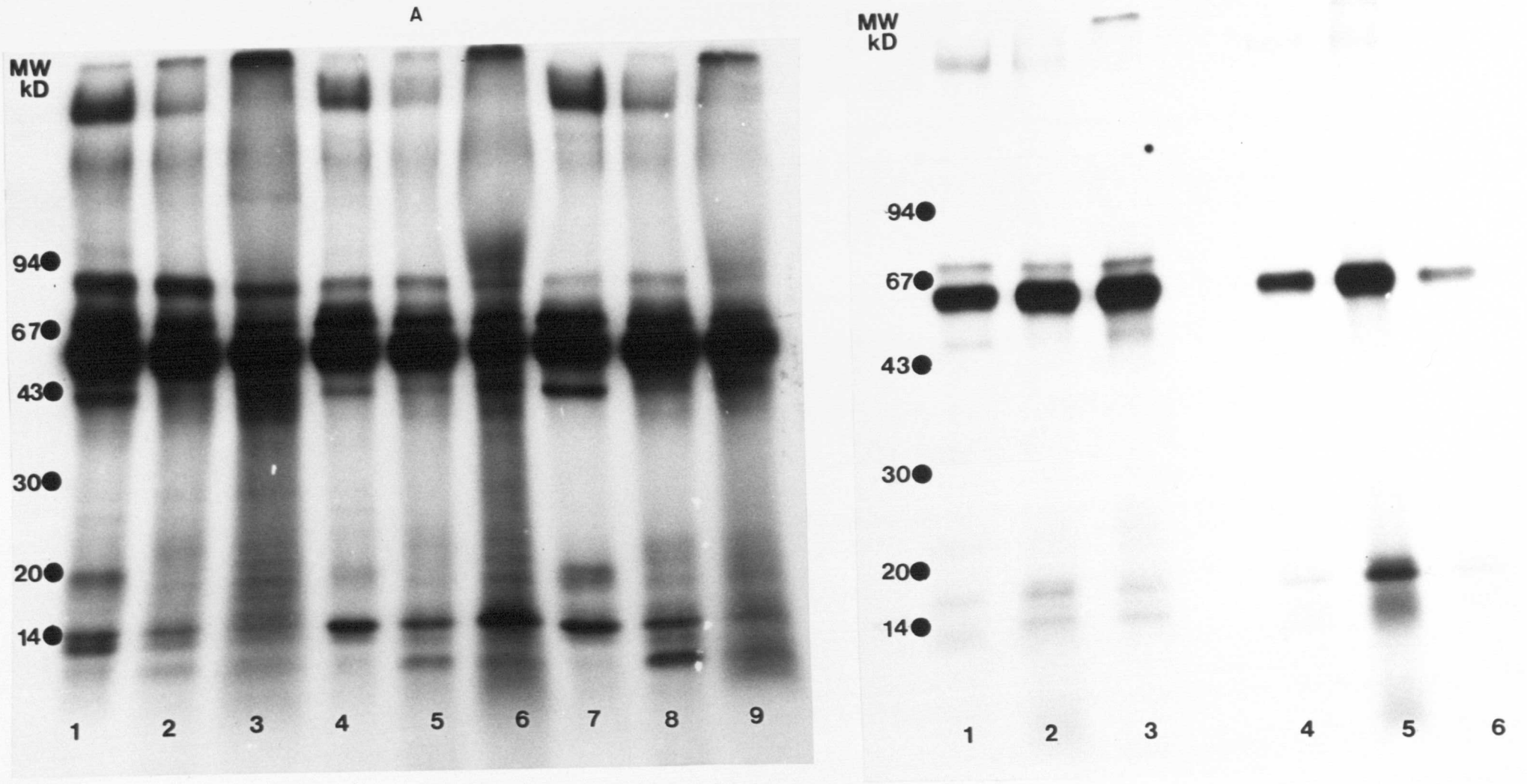
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Figure 4.5 Surface Protein Profile of Parasites Aged Between Days 1-5

Parasites were radio-iodinated using the Iodo-gen method and then the molecules were extracted firstly using PBS (S1) and then SDS (S2) and finally B-ME (S3). 10,000 cpm of each extract were run under reducing conditions on a 5-20% SDS-PAGE gel and the gel autoradiographed. The molecular weight standards are shown on the left hand side.

(A)	Lane 1	Day 1 S1	(B)	Lane 1	Day 4 S1
	" 2	Day 1 S2		" 2	Day 4 S2
	" 3	Day 1 S3		" 3	Day 4 S3
	" 4	Day 2 S1		" 4	Day 5 S1
	" 5	Day 2 S2		" 5	Day 5 S2
	" 6	Day 2 S3		" 6	Day 5 S3
	" 7	Day 3 S1			
	" 8	Day 3 S2			
	" 9	Day 3 S3			

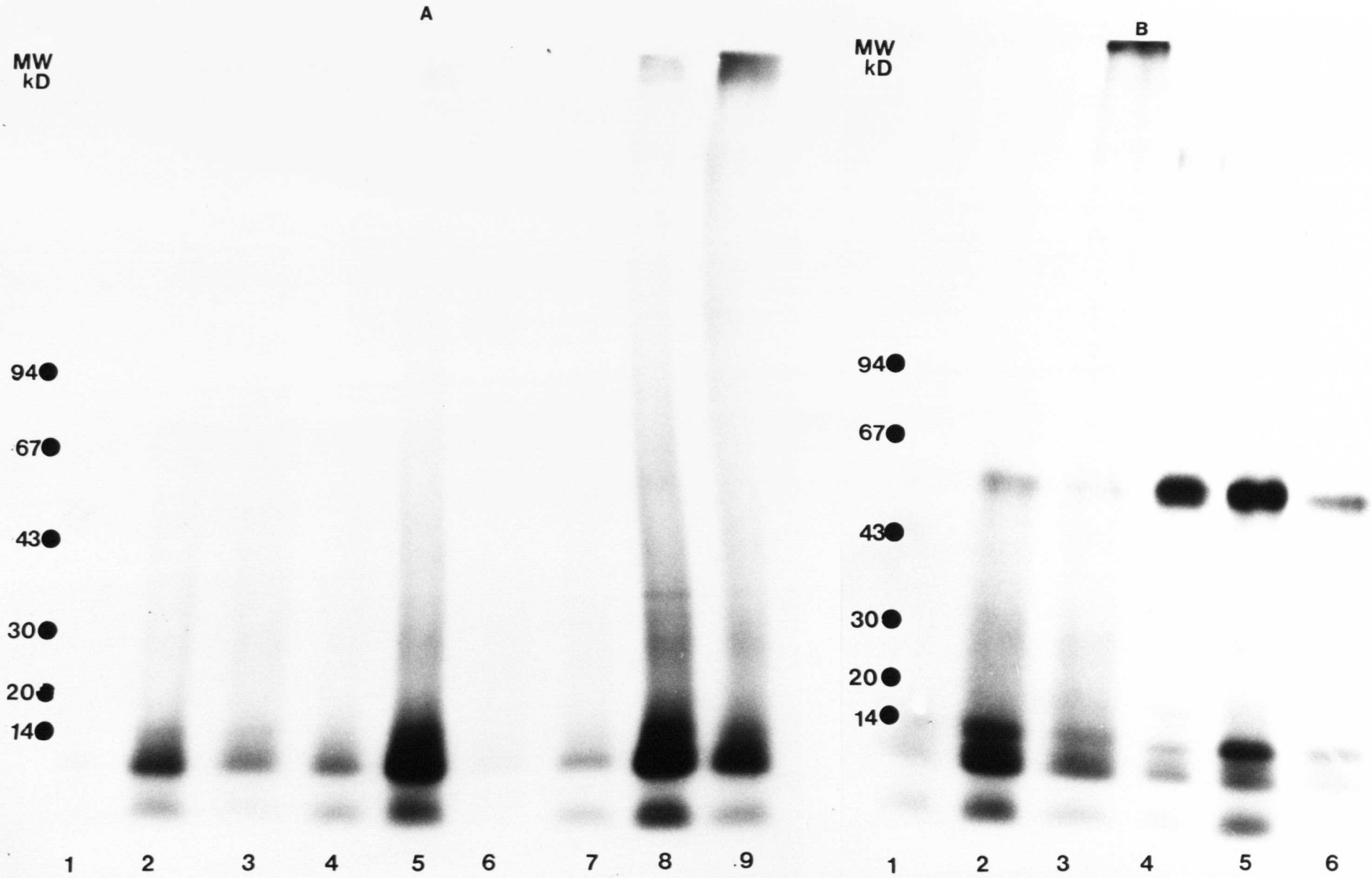
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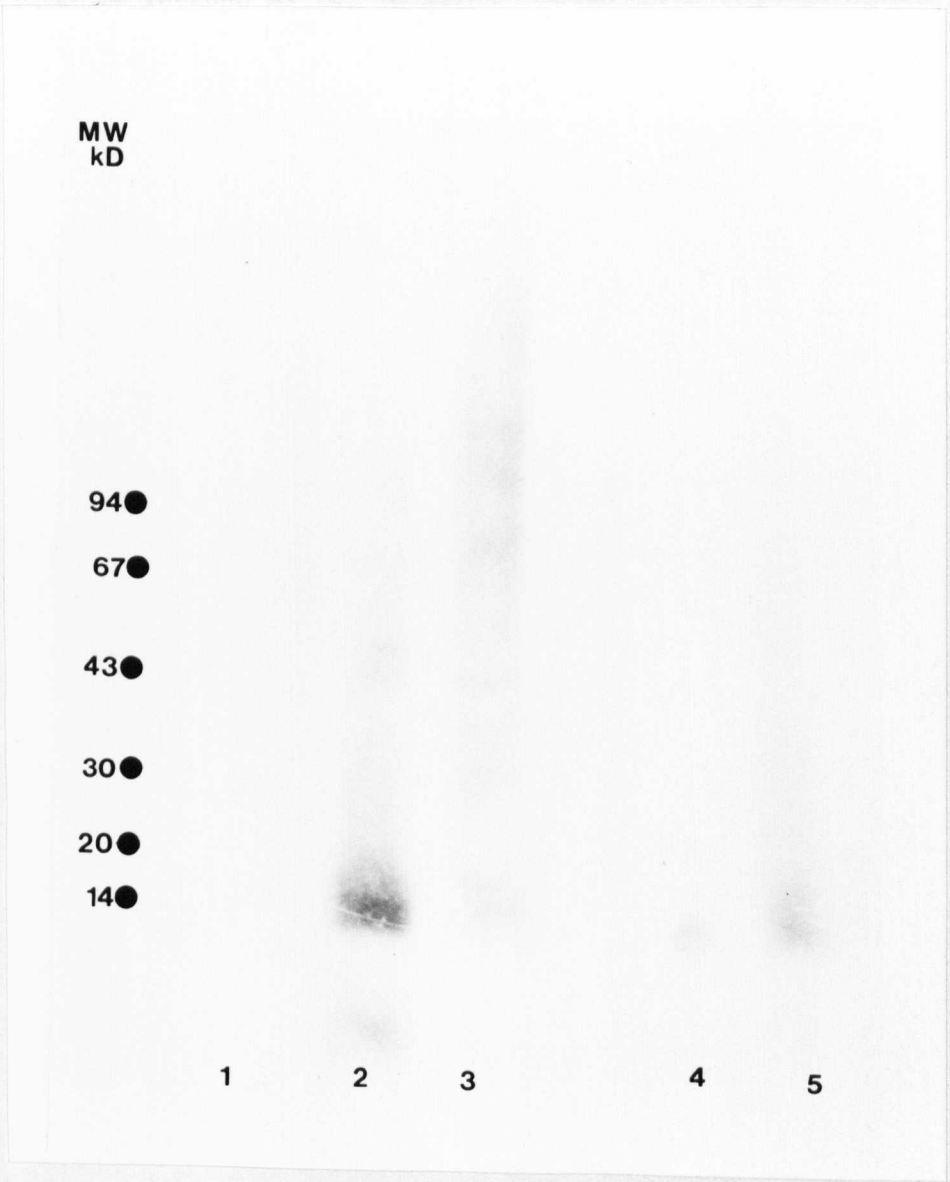
Figure 4.6 Surface Protein Profile of L3 Parasites

Parasites were radio-iodinated using the Iodo-gen method and then the molecules were extracted firstly using PBS (S1) and then SDS (S2) and finally B-ME (S3). 10,000 cpm of each extract were run under reducing conditions on a 5-20% SDS-PAGE gel and the gel autoradiographed. The molecular weight standards are shown on the left hand side.

Lane 1 L3 S1
" 2 L3 S2
" 3 L3 S3
" 4 L3 CTAB stripped supernatant
" 5 L3 Boiled Pellet of CTAB stripped Parasites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A summary of the molecules present on the various stages of the parasite and the preparation containing them is presented in Table 4.1. The bands present at 10 kDa may represent a number of molecules as those at day 1 seem to extracted in a different way to those at later stages. There is also an absence of the molecule at the late L4 stage but it reappears by day 8.

Table 4.1 Cuticle Solubilisation of Parasites

The major molecules present in extracts of the various stages of surface iodinated parasites were calculated from Figs. 4.2, 4.4, 4.5 and 4.6 and summarised in the table.

S1: molecules extracted by PBS,
S2: molecules extracted by SDS,
S3: molecules extracted by B-ME.

Stage	Molecules Present (kDa)
Adult Male	237 - - 76 - - - -
Adult Female	232 145 - - - - 20?15?
Day 10	- 208 145 92 76 62 47 20 15 - 10
Day 9	- 208 145 92 76 62 47 20 15 - 10
Day 8	- 208 145 92 76 62 47 20 15 - 10
Day 7	- 208 - - 76 62 47 - 15 12 -
Day 6#	- 208 - - - 62 - - 15 12 -
Day 5	335 - - - - 62 - - 15 12 10
Day 4*	- - - - - 62 - - 15 12 10
Day 3	- - - - - - - 32 - 12 10
Day 2	- - - - - - - - - 12 10
Day 1	- - - - - - - - - $12^{2,3}$ 10^{2}
L3	- - 115^{3} - 74^{3} - 48^{2} 40^{3} $14^{2,3}$ $7^{2,3}$

N.B. * Moult from L3 to L4
Moult from L4 to adult
1 Present in S1
2 Present in S2
3 Present in S3

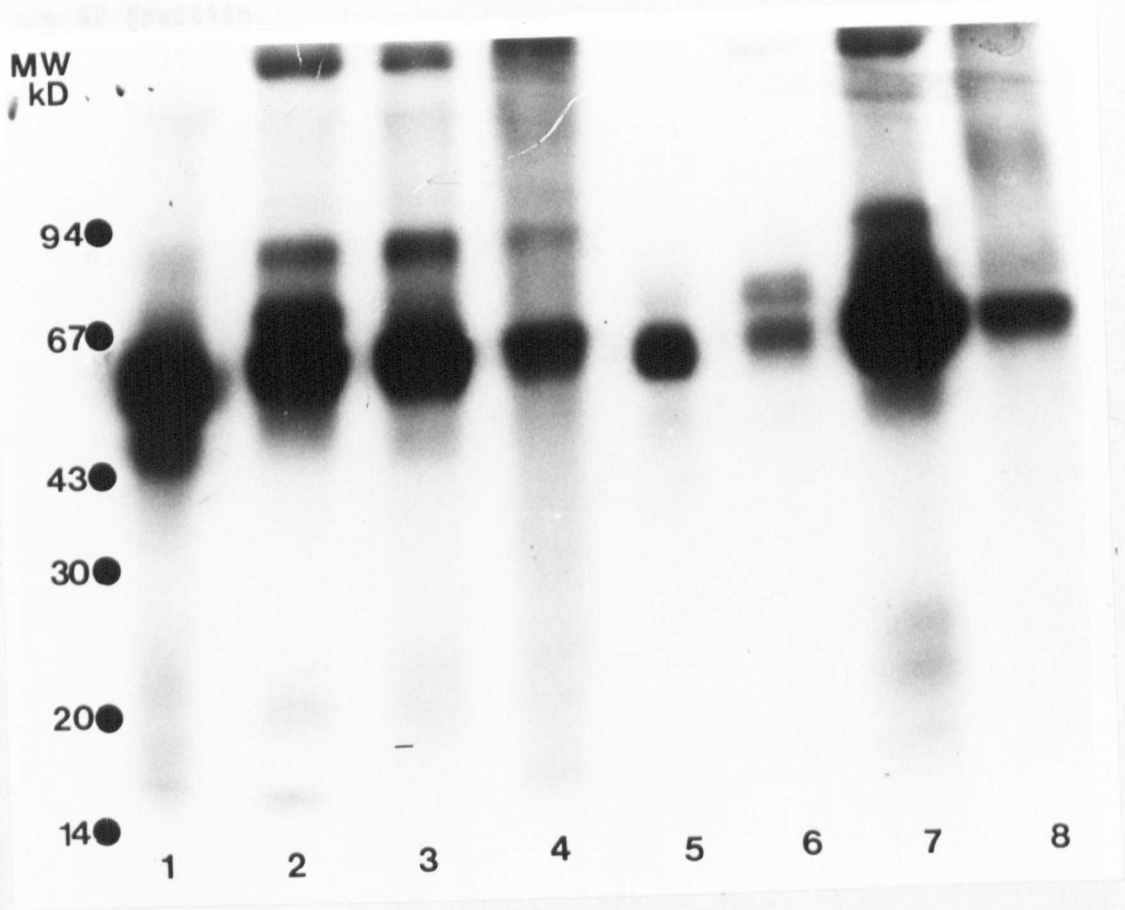
4.2.3 CTAB stripping

In an attempt to analyse the molecules present on the very outer surface, the glycocalyx, of the parasite, the worms were stripped using the cationic detergent CTAB. The effects of CTAB stripping days 9 and 10 p.i. are shown in Fig. 4.7, it appeared that the majority of the molecules present were removed through the action of this detergent, although the 92 kDa molecule was not removed to such a great extent as the other molecules. In the day 9 preparation, the only molecule that appeared to be removed was the 62 kDa, with the 76, 92, 208 and 20 kDa proteins being predominant in the S2 fraction.

Figure 4.7 CTAB Stripping of Surface Labelled Parasites Aged Between Days 9 and 10

Parasites were radio-iodinated using the Iodo-gen method and then the molecules from the surface were stripped using the cationic detergent cetyltrimethylammonium bromide (CTAB). After this treatment they were extracted firstly using PBS (S1) and then SDS (S2) and finally B-ME (S3). 10,000 cpm of each extract were run under reducing conditions on a 5-20% SDS-PAGE gel and the gel autoradiographed. The molecular weight standards are shown on the left hand side.

Lane	1	Day 10	CTAB	Stripped	Supernatant
"	2	Day 10	"	"	S1
"	3	Day 10	"	"	S2
"	4	Day 10	"	"	S3
"	5	Day 9	"	"	Supernatant
"	6	Day 9	"	"	S1
"	7	Day 9	"	"	S2
"	8	Day 9	"	"	S3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Days 7 and 8 (Fig. 4.8a) also showed a more restricted profile. Again, only the 62 kDa molecule was stripped from the parasite surface, with the majority of bands being present in the S2 fraction of the preparations. The day 6 parasites (Fig. 4.8b) also showed a limited profile but this may also be because there was demonstrated to be fewer molecules present at this stage. Two molecules were removed (62 and 20 kDa) whereas the 208 and 15 kDa molecules remained but were only present in the S2 fraction.

Figure 4.8 CTAB Stripping of Surface Labelled Parasites Aged Between Days 6-8

Parasites were radio-iodinated using the Iodo-gen method and then the molecules from the surface were stripped using the cationic detergent cetyltrimethylammonium bromide (CTAB). After this treatment they were extracted firstly using PBS (S1) and then SDS (S2) and finally B-ME (S3). 10,000 cpm of each extract were run under reducing conditions on a 5-20% SDS-PAGE gel and the gel autoradiographed. The molecular weight standards are shown on the left hand side.

(A)	Lane	1	Day 8	CTAB	Stripped	Supernatant
	"	2	Day 8	"	"	S1
	"	3	Day 8	"	"	S2
	"	4	Day 8	"	"	S3
	"	5	Day 7	"	"	Supernatant
	"	6	Day 7	"	"	S1
	"	7	Day 7	"	"	S2
	"	8	Day 7	"	"	S3

(B)	Lane	1	Day 6	CTAB	Stripped	Supernatant
	"	2	Day 6	"	"	S1
	"	3	Day 6	"	"	S2
	"	4	Day 6	"	"	S3

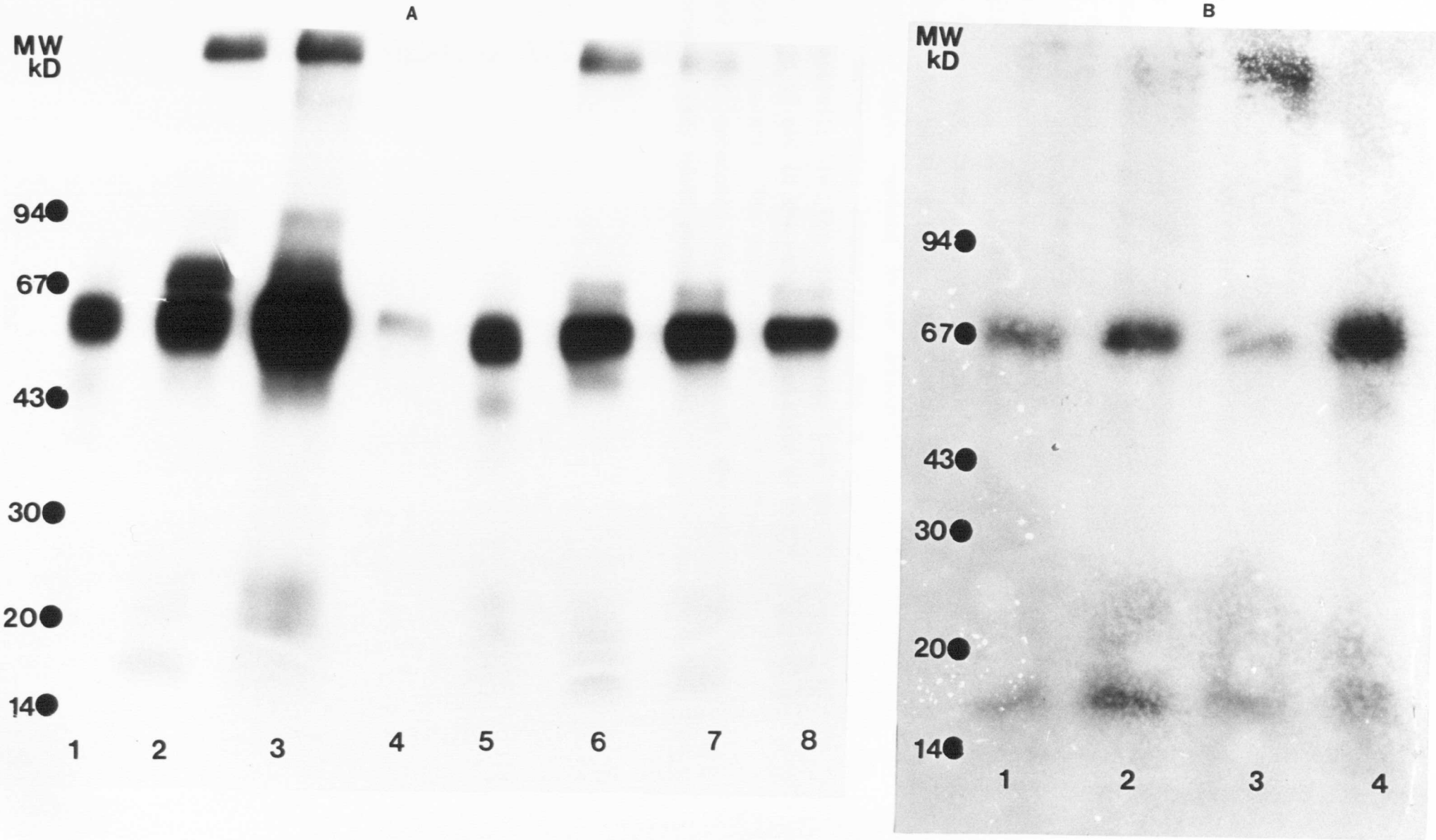
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Fig 4.9 shows the effect of CTAB stripping days 1-5, unfortunately, due to the lack of available material a cuticle preparation could not be done on the parasites after they had been subjected to the stripping procedure, and so the parasites were only homogenised in a SDS/B-ME buffer in order to extract as many molecules as possible in one sample. CTAB stripping of the day 1 worms did not reveal any molecules in the CTAB supernatant, although molecules at 62 and 20 kDa were visible in the cuticle homogenate. Day 2 revealed molecules at 12 and possibly 10 kDa, with only the 62 kDa protein being observed in the homogenate, thus, the majority of the molecules on the surface must be strippable. Day 3 worms also showed the same molecules being removed by the detergent, with the 15 kDa protein being very concentrated. Day 4 also demonstrated little activity in the CTAB fraction, but those molecules present at 62 and 15 kDa were representative of those observed in the homogenate. The supernatant of day 5 stripped parasites showed that molecules at 33, 45 and 10 kDa were present, although in very small amounts.

Figure 4.9 CTAB Stripping of Surface Labelled Parasites Aged Between Days 1-5

Parasites were radio-iodinated using the Iodo-gen method and then the molecules from the surface were stripped using the cationic detergent cetyltrimethylammonium bromide (CTAB). After this treatment they were extracted by boiling in reducing sample buffer. 10,000 cpm of each extract were run under reducing conditions on a 5-20% SDS-PAGE gel and the gel autoradiographed. The molecular weight standards are shown on the left hand side.

(A)	Lane 1	Day 1	CTAB	Stripped	Supernatant
	" 2	Day 1	"	"	Boiled Pellet
	" 3	Day 2	"	"	Supernatant
	" 4	Day 2	"	"	Boiled Pellet
	" 5	Day 3	"	"	Supernatant
	" 6	Day 3	"	"	Boiled Pellet
	" 7	Day 4	"	"	Supernatant
	" 8	Day 4	"	"	Boiled Pellet
(B)	Lane 1	Day 5	"	"	Supernatant
	" 2	Day 5	"	"	Boiled Pellet

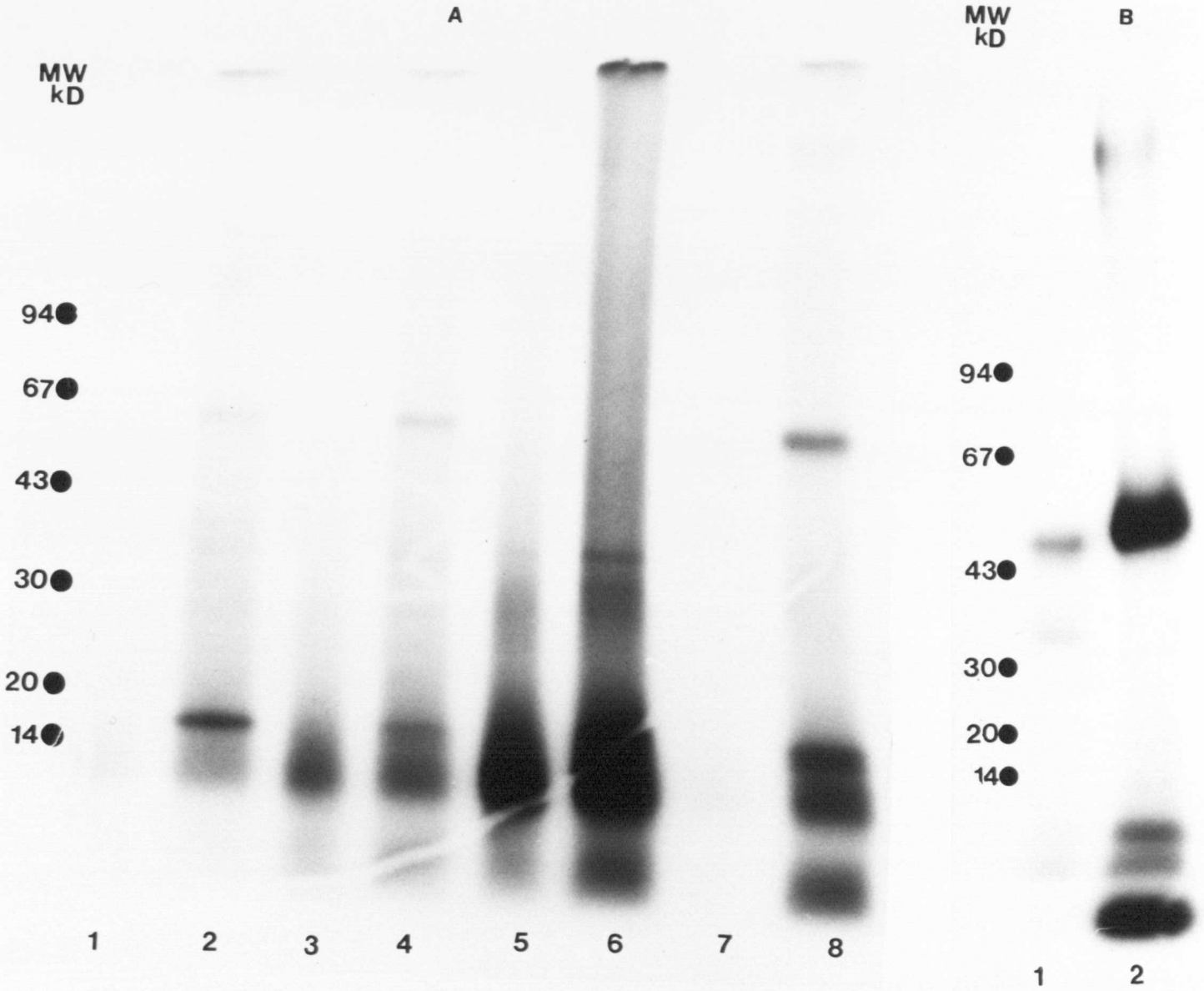
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Table 4.2 shows a summary of the molecules present in the CTAB supernatant and the remaining fractions of the parasite after the various stages have been stripped with the cationic detergent, CTAB.

Table 4.2 Cuticle Solubilisations of CTAB Stripped Parasites

Various stages of the parasites were radio-iodinated and then the surface molecules removed using the cationic detergent, CTAB. After the surface molecules were stripped, the remaining molecules were extracted using the cuticle solubilisation technique. The major molecules present in the CTAB supernatant and in the solubilised cuticles were calculated from Figs. 4.6, 4.7, 4.8 and 4.9 and are summarised in the table.

CTAB S/N: molecules stripped by CTAB,
S1: molecules extracted by PBS,
S2: molecules extracted by SDS,
S3: molecules extracted by B-ME.

Molecules Present (kDa)

Preparation

Stage	CTAB S/N						S1/S2/S3											
Day 10	62	47	20	15	-	-	-	-	145	-	92	76	62	-	20	15		
Day 9	62	47	-	-	-	-	-	-	145	-	92	76	62	-	20	15		
Day 8	62	-	-	-	-	-	-	188	-	-	92^{2}	76^{1}	62	47	20	-		
Day 7	62	47	-	-	-	-	-	188	-	-	-	76	62	47	-	-		
Day 6#	62	-	-	15	-	-	-	188	-	-	-	-	62	-	-	15		
Day 5	62	-	-	-	-	-	335	-	-	-	-	-	62	-	-	-	12	7
Day 4*	62	-	-	-	-	-	-	-	-	-	-	-	62	-	-	15	12	7
Day 3	-	-	-	15	12	7	-	-	-	-	-	-	-	32	-	15	12	7
Day 2	-	-	-	-	12	7	-	-	-	-	-	-	62	-	-	15	12	7
Day 1	-	-	-	-	-	-	-	-	-	-	-	-	62	-	-	15	12	-
L3	-	-	-	15	-	-	-	-	-	115	-	-	-	40	-	15	-	-

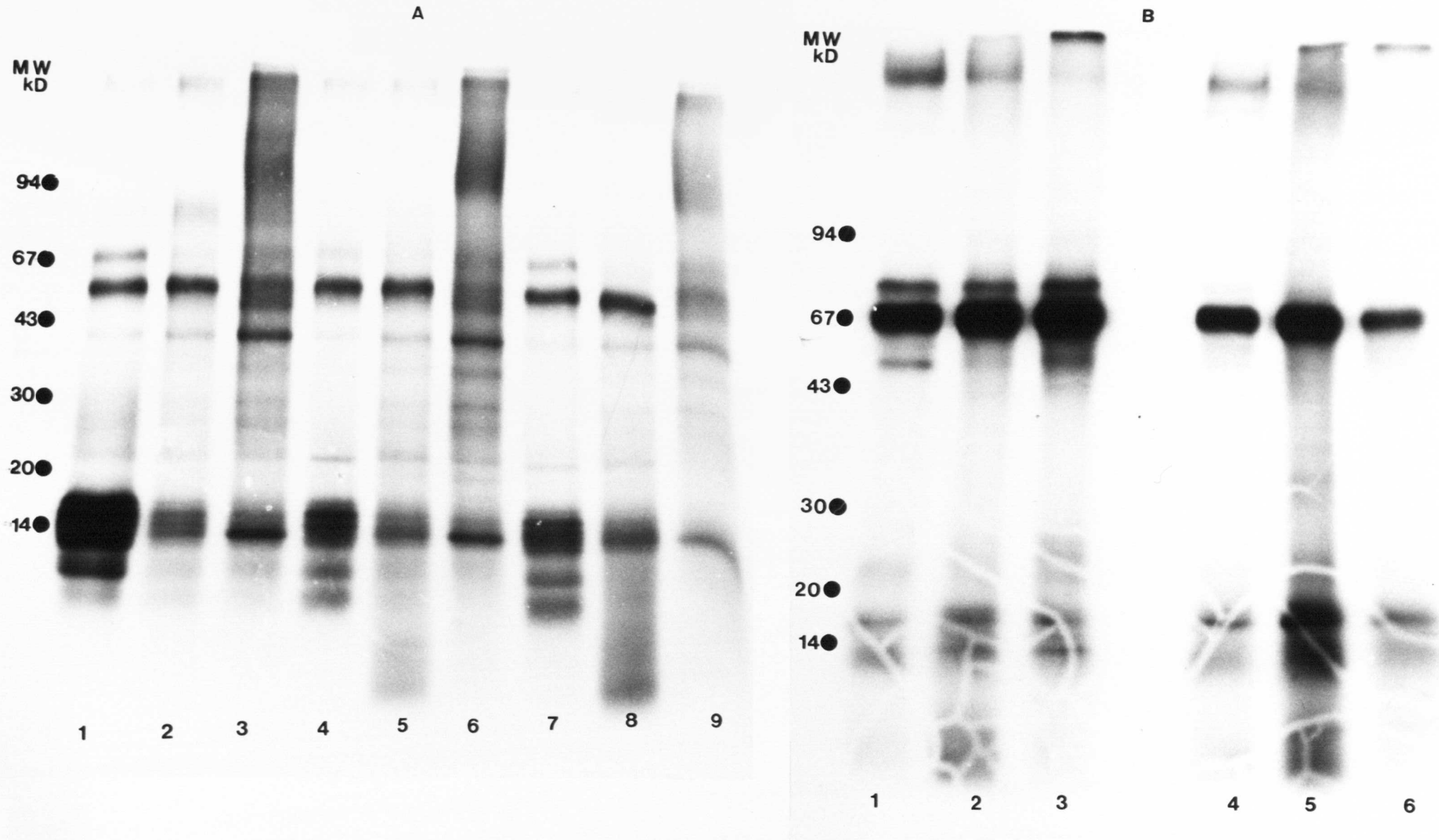
N.B. * Moult from L3 to L4
Moult from L4 to adult
[1] Present in S1
[2] Present in S2

CTAB stripping prior to the labelling procedure allows radio-iodination to occur in deeper layers of the cuticle and so, may reveal molecules which were previously undetected. In this experiment only parasites from days 6-10 were used as insufficient material could be obtained from the earlier stages. It was seen (Fig. 4.10) that not only did CTAB stripping prior to labelling allow a greater number of molecules to be labelled but they were also radio-iodinated to a larger extent. Extra molecules were labelled at 17, 16 and 10 kDa. Interestingly, the 76, 62 and 47 kDa proteins were not as predominant in each preparation as in previous gels, thus suggesting that the majority of these molecules had been removed by the detergent. The 15 kDa band was the predominant molecule and labelled extremely strongly, implying that it was the dominant molecule beneath the glycocalyx. It was also interesting to note that a 10 kDa molecule was more apparent in these preparations. The S2 fractions from days 8 and 9 also appeared to have a faint band 6 kDa. CTAB stripping days 6 and 7 did not seem to reveal any extra molecules nor deplete those originally present, with the resulting profiles looking very similar for the stripped and unstripped preparations.

Figure 4.10 Surface Protein Profile of Parasites CTAB Stripped Prior to Labelling

The molecules from the surface of the parasites were removed using the cationic detergent cetyltrimethylammonium bromide (CTAB). They were radio-iodinated using the Iodo-gen method and then the remaining molecules were extracted firstly using PBS (S1) and then SDS (S2) and finally B-ME (S3). 10,000 cpm of each extract were run under reducing conditions on a 5-20% SDS-PAGE gel and the gel autoradiographed. The molecular weight standards are shown on the left hand side.

(A)	Lane 1	Day 10 S1	(B)	Lane 1	Day 7 S1
	" 2	Day 10 S2		" 2	Day 7 S2
	" 3	Day 10 S3		" 3	Day 7 S3
	" 4	Day 9 S1		" 4	Day 6 S1
	" 5	Day 9 S2		" 5	Day 6 S2
	" 6	Day 9 S3		" 6	Day 6 S3
	" 7	Day 8 S1			
	" 8	Day 8 S2			
	" 9	Day 8 S3			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A summary of the molecules labelled after days 6-10 of the parasite have been CTAB stripped is illustrated in Table 4.3.

Table 4.3 Cuticle Solubilisation of Parasites CTAB Stripped Prior to Labelling

Various stages of the parasites were radio-iodinated after the surface molecules had been removed using the cationic detergent, CTAB. The labelled molecules were extracted using the cuticle solubilisation technique and the weights of the major molecules present were calculated and are summarised in the table.

S1: molecules extracted by PBS,
S2: molecules extracted by SDS,
S3: molecules extracted by B-ME.

Stage	Molecules Present (kDa)										
Adult	208	92	76[1]	62	47	20	17	16[1,2]	15[1,2]	12[1]	10[1]
Day 10	208	92	76[1]	62	47	20	17	16[1,2]	15[1,2]	12[1]	10[1]
Day 9	208	92	76[1]	62	47	20	17	16[1,2]	15[1,2]	12[1]	10[1]
Day 8	208	92	76[1]	62	47	20	17	16[1,2]	15[1,2]	12[1]	10[1]
Day 7	208	92	-	62	47	-	17	-	15	-	10[2]
Day 6[#]	208	-	-	62	-	-	17	-	15	-	10[2]

N.B [#] Moult from L4 to adult
[1] Present in S1
[2] Present in S2
[3] Present in S3

4.2.4 Triton X 114 Extractions

In order to identify the hydrophobicity of the various molecules and thus their relationship to each other in the cuticle of the parasite, days 10-6 parasites were extracted using the detergent Triton X 114. A very restricted number of molecules were extracted using this procedure (Fig. 4.11, 4.12), with those molecules present being mainly found in the aqueous phase and a very limited number detected in the detergent phase. In days 9 and 10 the major molecules detected in the aqueous phase were 76, 62 and 20 kDa, whilst molecules at 62 and 20 were isolated from the detergent phase. This suggested that the 62 and 20 kDa molecules were amphiphilic and the 76 kDa molecule was hydrophilic. The samples ran in a slightly distorted fashion due to the presence of sucrose in the preparations. The preparations from days 7 and 8 also demonstrated a similar profile to the later stages, with molecules apparent in the aqueous phase at 76, 62, and 20 kDa and at 62 and 20 kDa in the detergent phase. Fig. 4.12 shows the profile Triton X 114 extracted day 6 parasites, again the resulting pattern for each preparation was very similar, with 2 molecules obvious in the detergent phase at 62 and 20 kDa.

Figure 4.11 Surface Labelled Parasites Extracted with the Detergent Triton X 114

Parasites were radio-iodinated using the Iodo-gen method and then hydrophilic and hydrophobic molecules were extracted using the detergent Triton X 114. 10,000 cpm of each extract were run under reducing conditions on a 5-20% SDS-PAGE gel and the gel autoradiographed. The molecular weight standards are shown on the left hand side.

(A) Lane 1 Day 10 Aqueous Phase
" 2 Day 10 Sucrose Cushion
" 3 Day 10 Detergent Phase
" 4 Day 10 Boiled Pellet
" 5 Day 9 Aqueous Phase
" 6 Day 9 Sucrose Cushion
" 7 Day 9 Detergent Phase
" 8 Day 9 Boiled Pellet

(B) Lane 1 Day 8 Aqueous Phase
" 2 Day 8 Sucrose Cushion
" 3 Day 8 Detergent Phase
" 4 Day 8 Boiled Pellet
" 5 Day 7 Aqueous Phase
" 6 Day 7 Sucrose Cushion
" 7 Day 7 Detergent Phase
" 8 Day 7 Boiled Pellet

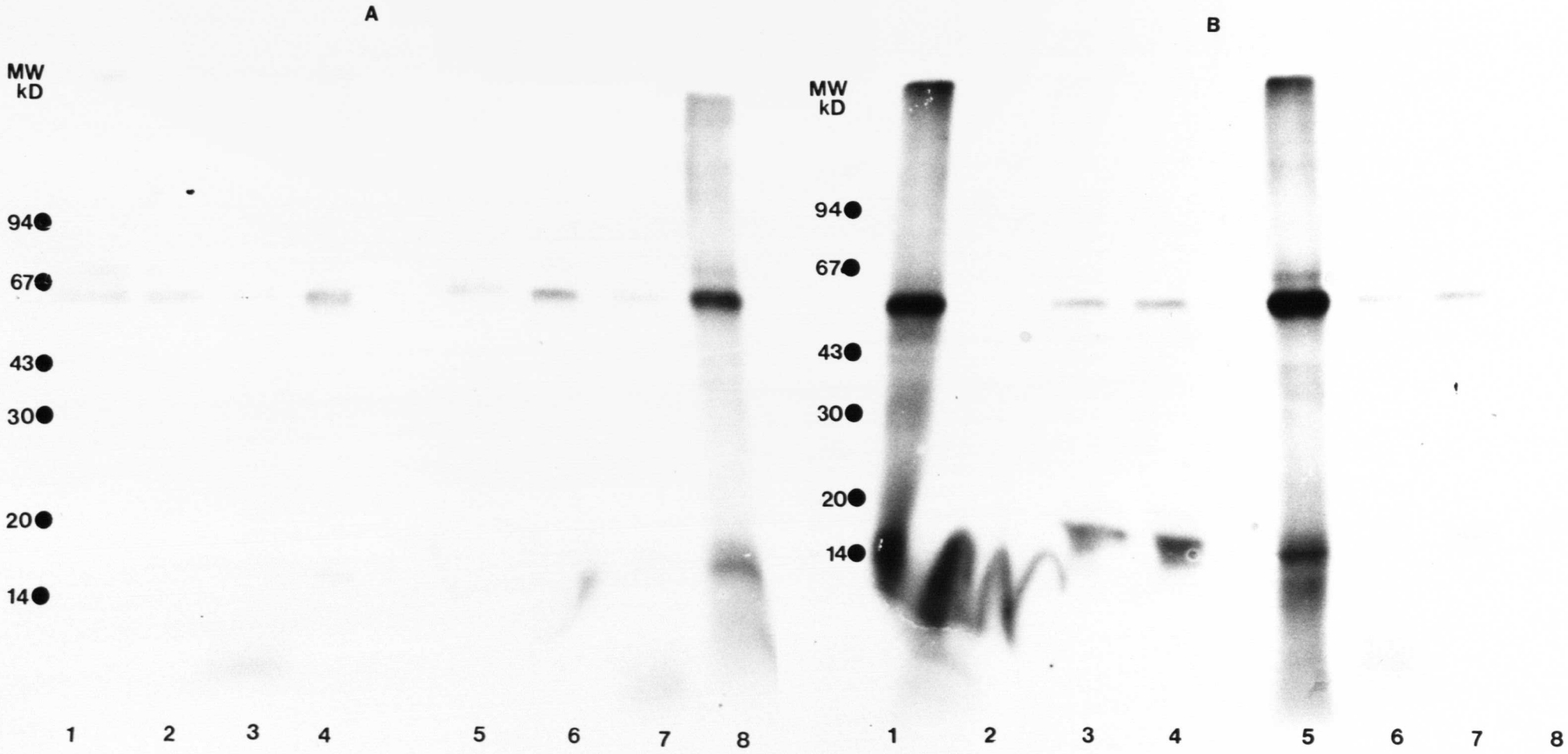
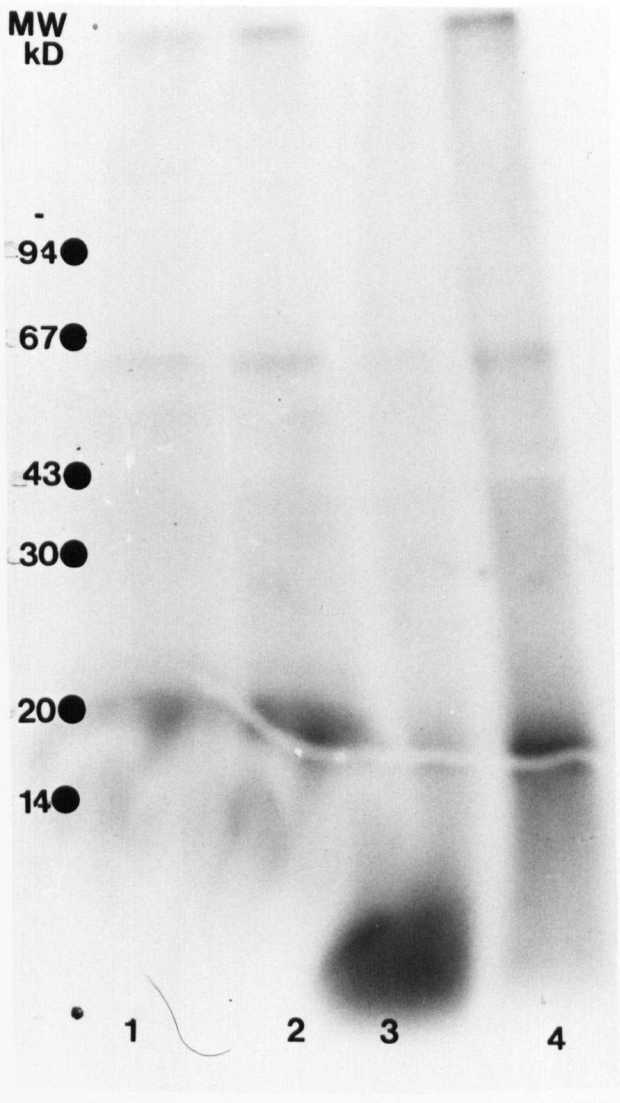

A
MW
kD
94
67
43
30
20
14
1
2
3
4
5
6
7
8
B
MW
kD
94
67
43
30
20
14
1
2
3
4
5
6
7
8

Figure 4.12 Surface Labelled Parasites Extracted with the Detergent Triton X 114

Parasites were radio-iodinated using the Iodo-gen method and then hydrophilic and hydrophobic molecules were extracted using the detergent Triton X 114. 10,000 cpm of each extract were run under reducing conditions on a 5-20% SDS-PAGE gel and the gel autoradiographed. The molecular weight standards are shown on the left hand side.

Lane 1 Day 6 Aqueous Phase
" 2 Day 6 Sucrose Cushion
" 3 Day 6 Detergent Phase
" 4 Day 6 Boiled Pellet


MW
kD
94
67
43
30
20
14
1
2
3
4

A summary of the molecules extracted in the aqueous and the detergent phases of Triton X 114 from parasites between days 6 and 10 p.i. is illustrated in Table 4.4.

Table 4.4 Triton X 114 Extraction of Labelled Parasites

Various stages of the parasite were radio-iodinated and the hydrophobic and hydrophilic molecules extracted using the detergent Triton X 114. The weights of the major molecules present were calculated and a summary is presented in the table.

Aqueous phase: Hydrophilic molecules
Detergent phase: Hydrophobic molecules

Molecules Present (kDa)

Stage	Preparation												
	Aqueous							Detergent					
Adult	208	145	92	76	62	47	20	92	62	47	-		
Day 10	-	-	-	76	62	-	20	-	62	-	20		
Day 9	-	-	-	76	62	-	20	-	62	-	20		
Day 8	-	-	-	76	62	-	20	-	62	-	20		
Day 7	-	145	-	76	62	47	20	-	62	-	20		
Day 6#	-	-	-	76	62	-	20	-	62	-	20		

N.B # Moult from L4 to adult

4.2.5 Quantitative Analysis of Labelling Procedures

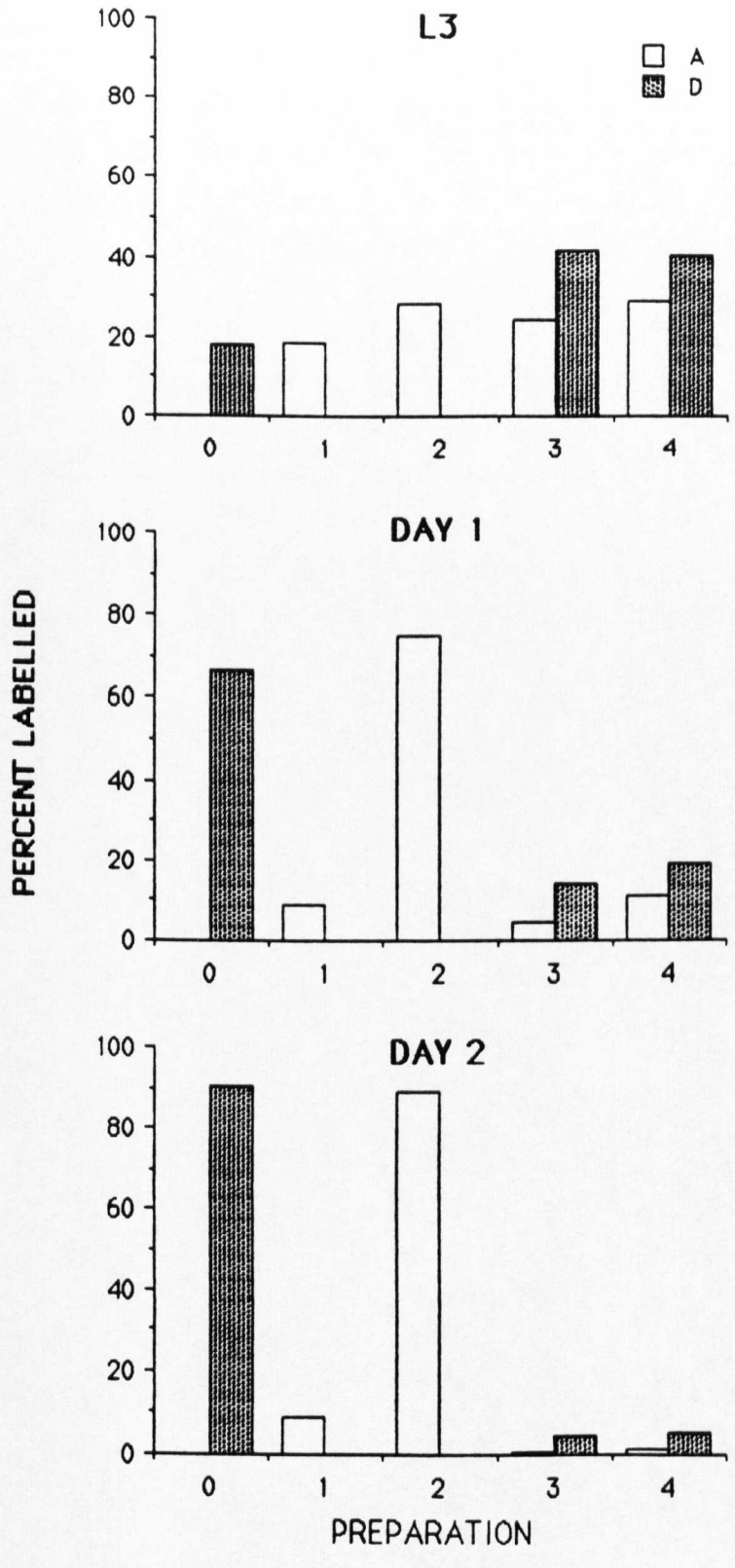
In order to quantify the various extraction procedures used on the labelled parasites, the percentage extracted for each preparation is shown. Fig. 4.13 demonstrated that the cuticle preparation of L3 worms resulted in each fraction being labelled equally, whereas CTAB stripping resulted in 20% of the counts being present in the detergent fraction, and the remainder being equally divided between the pellet and the homogenate. When days 1 and 2 were subjected to the same treatments, the patterns were quite different. In the cuticle preparations in both cases, the majority of the labelled material (80%), was found in the SDS fraction, with negligible amounts found in the remaining three fractions, including the pellet. When they were CTAB stripped, 80% of the labelled protein was found in the CTAB supernatant, whilst the remaining counts were divided equally between the homogenate and the pellet.

Figure 4.13 Percentage Incorporation of Label in Each Extraction

Parasites were radio-iodinated using the Iodo-gen method and the percentage incorporation of the label in each extraction was calculated.

Extraction method A: Cuticle solubilisation with PBS, SDS and B-ME. Extraction method D: CTAB stripping followed by boiling of the pellet in reducing sample buffer

Preparation	0	CTAB Stripped Supernatant
"	1	S1
"	2	S2
"	3	S3
"	4	Pellet


L3
A
D
DAY 1
DAY 2
PERCENT LABELLED
PREPARATION
100
80
60
40
20
0
0
1
2
3
4

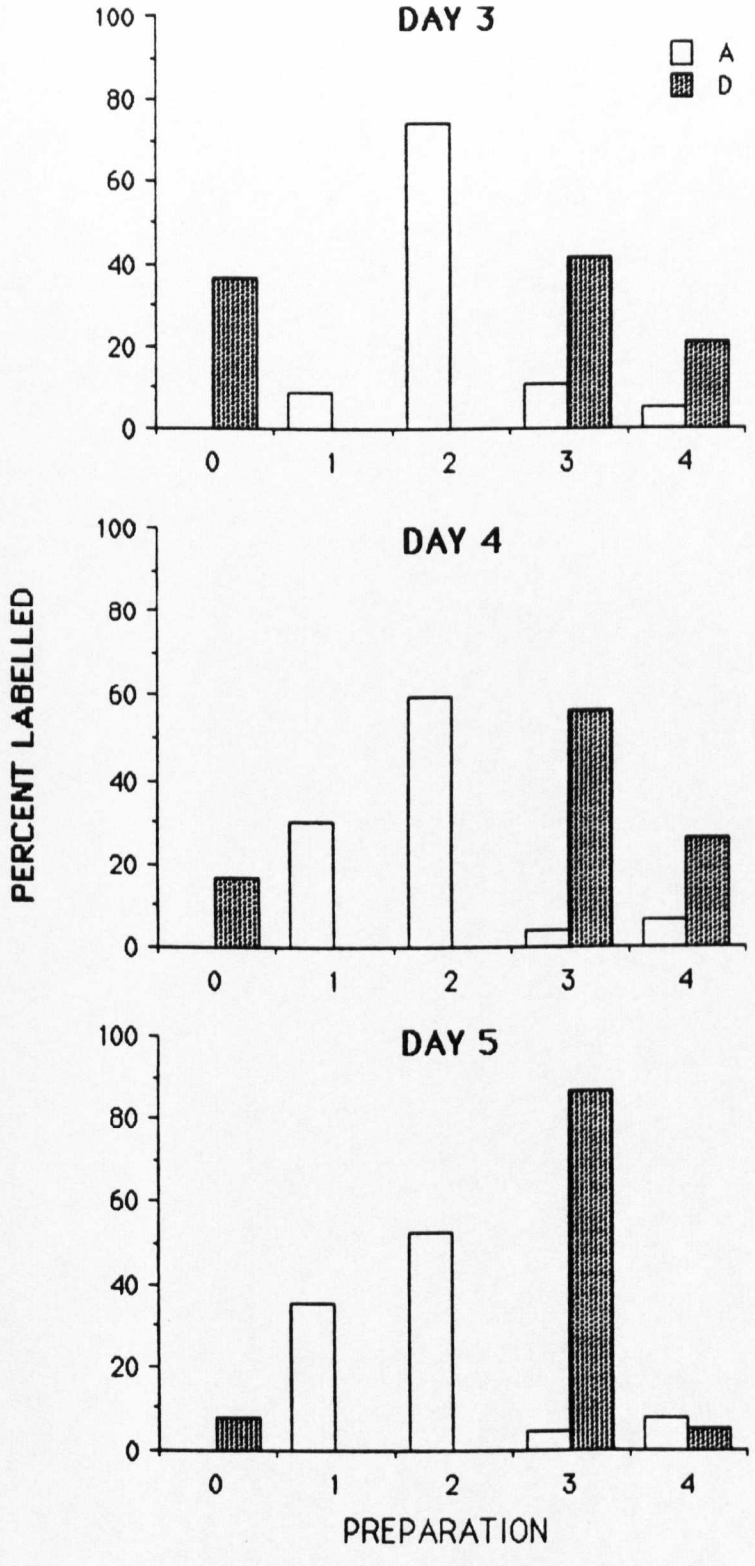
Day 3 parasites showed the same general pattern for the extraction of the cuticular molecules (Fig. 4.14), but the profile for the CTAB stripping was quite different. The CTAB supernatant only comprised of 40% of the counts, about half of that that was present in the same fraction on days 1 and 2, with the homogenate and pellet containing the majority of the labelled protein. In days 4 and 5 the change in this profile became more exaggerated. Although the S2 fraction still contained the majority of the material, the amount in S1 increased with age, however, the percentage found in the S3 fraction and pellet was still negligible. When these worms were CTAB stripped, a negligible proportion of the label was found in the supernatant (10%) compared with 80% for days 1 and 2. The greater portion of the labelled material was found in the homogenate, with the pellet containing very few counts.

Figure 4.14 Percentage Incorporation of Label in Each Extraction

Parasites were radio-iodinated using the Iodo-gen method and the percentage incorporation of the label in each extraction was calculated.

Extraction method A: Cuticle solubilisation with PBS, SDS and B-ME. Extraction method D: CTAB stripping followed by boiling of the pellet in reducing sample buffer

Preparation 0 CTAB Stripped Supernatant
" 1 S1
" 2 S2
" 3 S3
" 4 Pellet


DAY 3
A
D
100
80
60
40
20
0
0
1
2
3
4
DAY 4
PERCENT LABELLED
DAY 5
PREPARATION

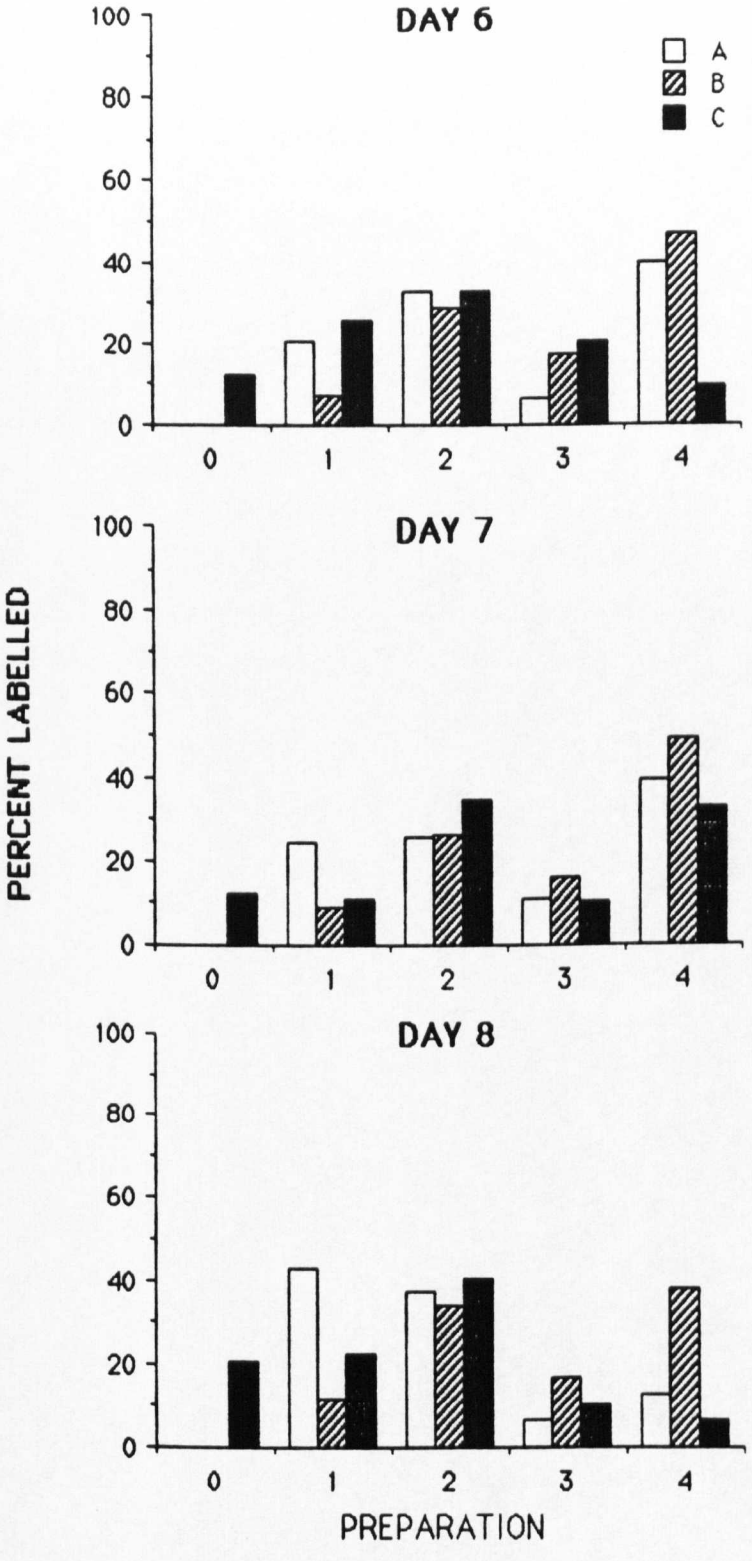
This profile altered even more in days 6-8 (Fig. 4.15). The cuticle preparation profile of days 6 and 7 was very similar with the S1 and S2 fractions accounting for similar amounts of the labelled protein. A large amount of the label was incorporated into the pellet (40%), but S3 accounted for less than 10% of the label. CTAB stripping the parasites prior to the labelling procedure resulted in a decrease in the PBS-soluble counts, whilst the detergent soluble counts remained unchanged. The label present in the S3 fraction increased considerably, as did that present in the pellet.

Figure 4.15 Percentage Incorporation of Label in Each Extraction

Parasites were radio-iodinated using the Iodo-gen method and the percentage incorporation of the label in each extraction was calculated.

Extraction method A: Cuticle solubilisation with PBS, SDS and B-ME. Extraction method B: Radio-iodination of the parasites after CTAB stripping, followed by cuticle solubilisation of the worms. Extraction method C: CTAB stripping of the parasites after radio-iodination followed by cuticle solubilisation.

Preparation 0 CTAB Stripped Supernatant
" 1 S1
" 2 S2
" 3 S3
" 4 Pellet


DAY 6
A
B
C
100
80
60
40
20
0
0
1
2
3
4
DAY 7
100
80
60
40
20
0
0
1
2
3
4
DAY 8
100
80
60
40
20
0
0
1
2
3
4
PERCENT LABELLED
PREPARATION

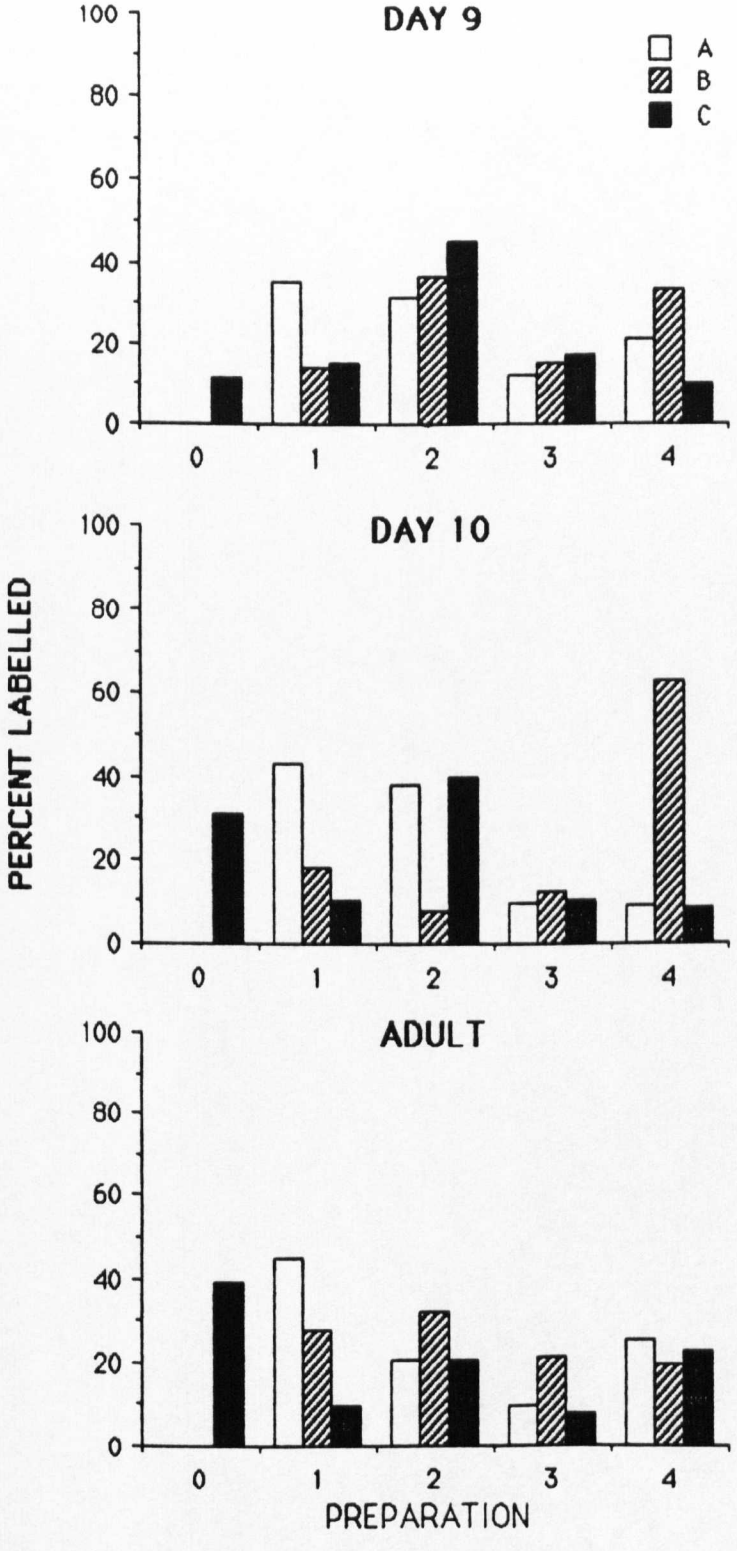
CTAB stripping prior to labelling again resulted in a decrease in the PBS-soluble counts, no change in the SDS and B-ME fractions and a fall in the number of pellet counts. Day 8 had a profile which was very similar to that of day 7, although the percentage of counts in the CTAB supernatant had increased. Days 9 and 10 (Fig. 4.16) again showed the same general pattern, with the CTAB fraction increasing the proportion of counts, and the amount incorporated into the pellet increasing after CTAB stripping. Day 30 adults showed a more exaggerated profile, the counts found in the PBS-soluble fraction decreased when the parasite was CTAB stripped before or after labelling. The counts in the SDS fraction did not alter appreciably, whilst the proportion of label in the B-ME fraction increased when the worms were CTAB stripped prior to labelling, however, there was no change in the percentage of counts present in the pellet. The proportion of counts present in the CTAB supernatant were increased.

Figure 4.16 Percentage Incorporation of Label in Each Extraction

Parasites were radio-iodinated using the Iodo-gen method and the percentage incorporation of the label in each extraction was calculated.

Extraction method A: Cuticle solubilisation with PBS, SDS and B-ME. Extraction method B: Radio-iodination of the parasites after CTAB stripping, followed by cuticle solubilisation of the worms. Extraction method C: CTAB stripping of the parasites after radio-iodination followed by cuticle solubilisation.

Preparation	0	CTAB Stripped Supernatant
"	1	S1
"	2	S2
"	3	S3
"	4	Pellet

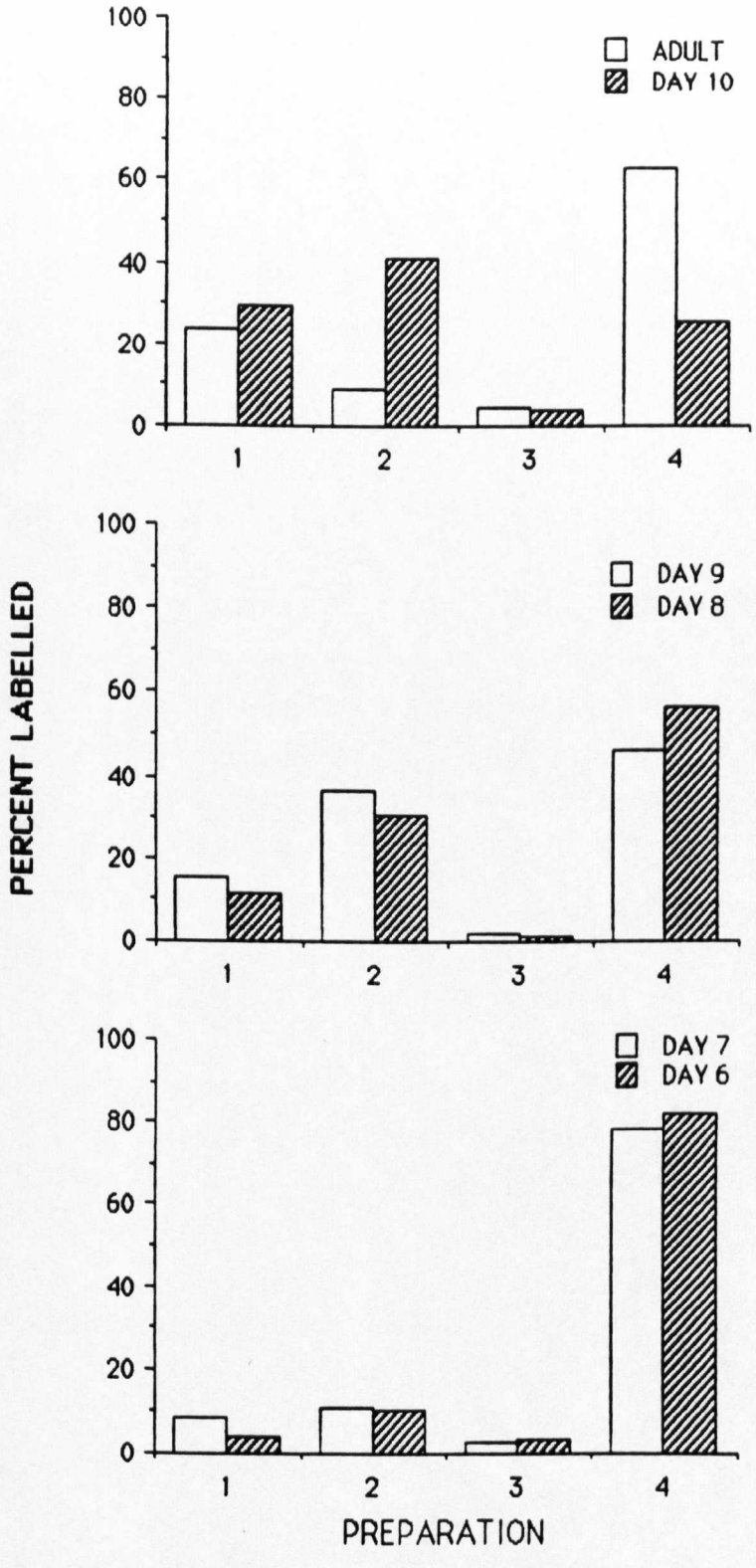

DAY 9
A
B
C
100
80
60
40
20
0
0
1
2
3
4
DAY 10
PERCENT LABELLED
ADULT
PREPARATION

When the Triton X 114 extraction profiles were examined, there was a totally different pattern apparent. In all the stages tested, there was negligible activity in the detergent fraction and maximum activity in the pellet. The proportion of counts found in the aqueous phase increased as the parasite matured.

Figure 4.17 Percentage of Incorporation of Label in Each Extraction

Parasites were radio-labelled using the Iodo-gen method and the percentage incorporation in each Triton X 114 extraction calculated.

Preparation 1 Aqueous Phase
" 2 Sucrose Cushion
" 3 Detergent Phase
" 4 Pellet


100
80
60
40
20
0
ADULT
DAY 10
1
2
3
4
DAY 9
DAY 8
DAY 7
DAY 6
PERCENT LABELLED
PREPARATION

4.3 DISCUSSION

Overall, it was seen that the proteins present in the cuticle of the nematode *H. polygyrus* altered profoundly during the development of the parasite from the infective third larval stage through to the tissue-dwelling fourth larval stage to a mature adult worm in the hosts intestinal lumen. These changes probably represent the changing relationship between the parasite and its environment. To begin with, the parasite is a free-living non-feeding form, and probably requires the cuticle to be a rather inert structure in order to protect it from the harsh environment. When the parasite enters the host the cuticle must be able to protect the parasite from both the acidic environment of the host stomach and the digestive enzymes present both there and in the duodenum. However, it needs to remain permeable to allow both pH and the concentrations of both oxygen and carbon dioxide to be detected by the worm in order to provide the signals for exsheathment. As the parasite develops and matures the cuticle needs to be able to protect the worm from both host enzymic activity and immune responses.

4.3.1 Preliminary Extractions

It can be seen that initial attempts to extract cuticular proteins from both adult and exsheathed L3 were largely unsuccessful, with only a limited number of molecules being distinguishable on the gels, and the majority of the labelled protein being retained in the pellet. A large number of proteins appeared on the exsheathed larvae but none were apparent on their cast sheaths. This suggested that the sheath is a rather inert structure, with few proteins amenable to the labelling procedure. Attempts to label infective larvae of other parasites such as *N. americanus*, *A. ceylanicum* and *N. brasiliensis* have also been unsuccessful despite the use of a number of extraction procedures and labelling methods. This implied that either there were few tyrosine molecules on the

parasite cuticle (Iodo-gen labels tyrosine residues) or that the surface proteins were being masked in some way, perhaps by carbohydrate molecules, which impeded the penetration of the label. Treatment with sodium periodate removes carbohydrate molecules and so would clarify this.

Studies by McKean, Pritchard and Rogan (1989) demonstrated that extraction of *N. americanus* cuticles using PBS, SDS and B-ME resulted in a protein profile which allowed cuticular proteins to be analysed and collagenous material to be isolated. Therefore, it was decided to use this extraction procedure in all subsequent experiments.

Preliminary experiments were performed in order to see if this extraction procedure could identify sex specific proteins, and if so, their relative location in the cuticle. Adams, East, Monroy and Dobson (1988) illustrated the presence of sex specific proteins on the surface of *H. polygyrus*. A male specific molecule 66 kDa protein was found on both the surface and in the ES of adult parasites. A 180 kDa female specific molecule was found on the surface of female worms, whilst a 30 kDa molecule was present in female ES. In the present study, a 70 kDa male specific and a 145 kDa female specific proteins were noted on the surface of the parasites which could correspond to those molecules illustrated in the other study. Molecules at 15 and 20 kDa were also possibly female specific. Other authors have noted sex specific molecules in other parasitic nematodes e.g. *N. brasiliensis* (Maizels, Meghje and Ogilvie, 1983) and *N. americanus* (Pritchard, Behnke, Carr and Wells, 1986). The function of these molecules is not clear but it is thought that they may be involved in a number of activities including, chemoattraction and mating. Molecular specialisation on the copulatory organ of *T. spiralis* has been demonstrated (Ortega-Pierres, Clark and Parkhouse, 1985) whilst a male pheromone receptor has been detected on the surface of *T. colubriformis* (Bone and Bottjer, 1985). Alternatively, female associated proteins could be a result of contamination with parasite egg proteins since female adult *H. polygyrus* contain large numbers of eggs. Labelling and extraction of

purified parasite eggs would confirm this hypothesis.

The use of detergents allows for greater dissection of the nematode cuticle. The epicuticle consists of a trilamellate layer covered by a polyanionic glycocalyx (Lee, 1977; Lumsden, 1975; Inglis, 1983). The epicuticle is known to bind polyvalent cationic complexes such as ruthenium red and cationized ferritin (Murrel and Graham, 1983; Himmelhoch and Zuckerman, 1983) and so it is thought that the net surface charge is negative. Therefore, in order to selectively solubilise the nematode surface, a cationic detergent was used to strip molecules from the surface of the parasite. Cetylmethylammonium bromide (CTAB) has been shown to strip appreciably more protein from the surface of *H. polygyrus* compared with other detergents (Pritchard, Crawford, Duce and Behnke, 1985) and so this procedure was adopted for the investigation of molecules from the epicuticular complex. It was demonstrated that 67% of the total available labelled material was removed compared to 19-22% with other detergents such as Triton X 100, Empigen BB and sodium deoxycholate (non-ionic, zwitterionic and anionic detergents). Surface labelled material was also shown to be effectively released from day 6 larval *H. polygyrus* suggesting that the net charge of the surface was similar in adult and larval parasites.

Preliminary studies on adult parasites suggested that the majority of the labelled material was present in the CTAB supernatant, although the low molecular weight material was not removed. The molecule at 208 kDa appeared to be preferentially removed. CTAB stripping the parasites prior to the labelling procedure may have exposed and labelled molecules which were previously masked by other molecules. It was noted that this procedure altered the profile of the molecules labelled, in that, those that were most strongly iodinated were not following CTAB stripping. This implied that the majority of the surface molecules had been removed. No additional molecules appeared to have been labelled using this technique, although the 17 kDa molecule appeared to have been labelled more intensely, thus suggesting that a greater number of these

molecules have been exposed by the stripping procedure.

Triton X 114 is widely used in order to study hydrophobic proteins as they can be easily extracted using this detergent. This detergent was used in order to determine the hydrophobicity of the various surface proteins as some of these molecules may not be extracted using more conventional extraction methods due to their hydrophobic nature. These molecules may exist beneath the glycocalyx and form part of the structural matrix of the cuticle and thus maintain the integrity of the parasite. Exposure of these molecules during a host immune response could result in the disruption of the parasite cuticle and the expulsion of the parasite from the host.

From the results it was clear that the majority of the labelled material was present in the aqueous phase and sucrose cushion (contaminants from the aqueous phase). It was thought that the molecules in the detergent phase probably represented contaminants from the aqueous phase rather than truly hydrophilic molecules. However, it has been demonstrated (Pritchard, Maizels, Behnke and Appleby, 1984) that the 62 kDa molecule on the adult surface was a heterogenous molecule resolving at a number of isoelectric points. Thus, it was possible that one of these molecules is hydrophobic and so does not represent a contaminating protein. It was also present in higher concentrations than other molecules and the entire range of molecules known to be present on the parasite cuticle were not in the detergent phase, again implying that it is a truly hydrophilic molecule. Many of the molecules present remained in the pellet suggesting that this extraction procedure is not particularly efficient at extracting molecules from the parasite cuticle. However, the addition of SDS and B-ME will interfere with the Triton X 114 extraction by altering the charge of the molecule by causing conformational changes and so altering the hydrophobicity of the molecules.

4.3.2 Cuticle Preparations

When cuticle preparations of the various stages of the parasite were analysed, a striking stage specificity of expression of surface proteins was noted. Again, it appeared that the cuticle of the infective larvae was a rather inert structure. A very restricted range of parasite proteins were radio-iodinated. The proteins that did label did not appear to correspond with any of the molecules observed on the surface of exsheathed larvae. This implied that a substantial change occurred when the parasite exsheathed. Curiously, the sheaths from exsheathed larvae did not label at all suggesting that some change occurred even to the cast sheath. It is possible that the technique used to isolate molecules from the sheath was not as efficient as that used to identify the molecules present on ensheathed L3 and so not all the molecules present were revealed. As mentioned before, the sheath of the infective third stage larvae has to be fairly resilient in order to protect the parasites from a range of agents, including acidity and enzymic action, however it needs to be permeable enough to allow entry of oxygen, carbon dioxide and other molecules which act as stimuli for the exsheathment of the parasite.

As the parasite aged it was apparent that the surface protein profile became more complex. The 12 kDa protein present on days 1-3 appeared to be analogous to the 14 kDa molecule present on L3. The pattern between days 1 and 3 did not alter considerably, although the molecules did start to label more intensely and a molecule at 32 kDa briefly appeared. By day 4 the profile was much more complicated, with the appearance of a 62 kDa molecule which persisted until at least day 10. The dramatic change between days 3 and 4 could be accounted for by the fact that the worms moults from a L3 to a L4 at this time. This moult was demonstrated (see Chapter 3) to be accompanied by a rapid increase in size and the beginning of sexual differentiation associated with the appearance of the male copulatory bursa. The profile between days 4 and 6 did

not appear to change considerably, although the intensity of the 15 kDa molecule had decreased and a 208 kDa molecule had appeared. By day 7, there was, again, a change in the surface protein profile of the parasite. Although the 62, 15 and 12 kDa molecules were conserved, new molecules at 76 and 47 kDa appeared. Again, this dramatic change was associated with a parasitic moult, this time from L4 to adult. Thus, these molecules may be associated with the adult parasite. By days 7-8 the picture was even more complex, with the appearance of more protein molecules, but this may be due to a more efficient labelling technique. The differences appear to be associated with the loss of the 12 kDa protein, an increase in the 15 kDa molecule and the appearance of molecules at 20, 92 and 145 kDa. Although a large number of changes occurred they were not associated with a further parasitic moult. Instead this may be due to the change in the parasites environment as it migrates from the mucosa to the intestinal lumen. It may also be due to the sexual maturation of the parasite as it begins egg production.

Previous authors have also demonstrated stage specific proteins on the surface of *H. polygyrus*. Monroy, East, Dobson and Adams (1989) showed that a 60 kDa molecule was present both on the surface and in the secretions of the parasite from day 5 p.i. to adult. The 62 kDa molecule demonstrated in this study, which occurred from day 4 may represent the same molecule. It has also been shown that immune sera preferentially immunoprecipitates day 6 surface proteins (Pritchard, Maizels, Behnke and Appleby, 1984). A 16 kDa molecule was only precipitated from larval preparations but a 65 kDa protein was recognised in both larval and adult preparations although to a greater extent in the larval fraction. The 16 kDa molecule may be equivalent to the 12 kDa protein seen in days 1-7 parasites in this study, implying that this is associated with the late third and fourth larval stage of the parasite. The differential recognition of the 65 kDa protein from larval and adult stages was proposed to be the result of either a greater proportion of the molecule being present on the larval stage or

the presence of a heterogeneous group of molecules in that molecular weight range, a result of either different molecules being present, or differential glycosylation. It was noticeable in this study, that the early stages (days 4 and 5) the molecule was mainly present in the SDS fraction, whilst from day 7 onwards, it was found in increasing concentrations in the B-ME fraction. This implied that there may be two distinct molecules present, one which is SDS soluble and the other which is B-ME soluble and collagenous in nature. Pritchard and Carr (1987) demonstrated that both ova and larval homogenates were very heterogeneous, although a limited range of antigens were precipitated. Again, the major protein at 65 kDa was shown to be present both on the cuticular surface and in the ES. Here it was demonstrated that this molecule was heterogeneous, the group of adult molecules resolved at pI 4.0 and pI 5.85, whilst the larval molecules only resolved at pI 4.0. It was suggested that at least two different molecules were present at 65 kDa.

Adams, East, Monroy, Washington and Dobson (1987) stated that there were no major surface proteins between L3 and day 4 p.i., however, this may have been because they were using insufficient material. In this study, 5 mice were infected with 10,000 L3 for days 1-3 p.i., whereas they only used 1,000 L3 per animal. Even when they increased the dose to 10,000 L3 they still did not obtain sufficient material. This may have been due to the different recovery methods employed. In this study the mucosa of the entire small intestine was scraped off with a microscope slide, thus freeing encysted larvae trapped in the sub-mucosa, rather than relying on the movements of the larvae to free themselves from this layer. The previous authors demonstrated the presence of common antigens at 250, 60, 45 and 30 kDa, and adult specific molecules at 90 and 24/30 kDa. A L4 specific antigen was demonstrated at 24/30 kDa and L3 specific proteins were noted at 145 and 47 kDa. Again, it was illustrated that the adult 60 kDa molecule was a complex of several molecules at this weight. The only surface molecules were shown to be present at 65, 45 and 24/30 kDa, which

corresponded to some of the molecules demonstrated to be present on the surface of adult parasites in this study. It was also interesting to note that many more molecules were shown to be present on the surface of all the stages of the parasite than in previous studies.

Betshart and Jenkins (1987) using a similar extraction procedure suggested that the epicuticle of *D. viteae* was highly insoluble, as the greatest amount of labelled protein was found in the pellet. They proposed that the SDS fraction was composed of internal material and that of the SDS/B-ME fraction consisted of the cortical zone and the epicuticle. However, this study and others (Pritchard, Crawford, Duce and Behnke, 1985; Philipp and Rumjaneck, 1984) demonstrated that the molecules on the parasite surface were easily removed with detergents, including CTAB. Therefore, it is most likely that true surface proteins are being labelled using this procedure and somatic molecules were not accounting for the activity seen.

The fact that all three cuticle preparations in each of the stages were very similar may be attributed to contaminating proteins from the preceding extraction. However, the method used was identical to that employed by Pritchard, McKean and Rogan (1988) where they found quite different molecules in each fraction. Each wash was counted and it was seen that immediately prior to the next extraction they represented 1% of the total counts whereas the the extraction accounted for about 30%. Thus the material extracted at each stage was entirely due to the action of SDS or B-ME and not merely that leached out by a washing procedure. It has been further demonstrated that this technique confines the label to the surface using both antigen stripping and microscopical techniques (Fraker and Speck, 1978; Pritchard, Crawford, Duce and Behnke, 1985, Parkhouse, Philipp and Ogilvie, 1981; Pritchard, McKean and Rogan, 1988)

4.3.3 CTAB stripping

CTAB stripping parasites reveals what molecules are on the very surface, i.e. the glycocalyx, and not those that are in the deeper lying areas of the cuticle but which are still susceptible to surface labelling. From our investigations it was noted that a very restricted range of molecules were removed from the parasite surface between days 1 and 4. These were the 15 kDa protein in the earlier stages but by day 4 they included the 62 kDa molecule. It appears that, once again, this band represents a complex number of proteins as the CTAB stripped molecule appears to come from the lower portion of this band, molecules in the upper portions of this 62 kDa band do not seem to be removed by the detergent. By day 2, an increasing proportion of this band could be removed by CTAB and by day 8, this was the only band that was present in the CTAB supernatant, suggesting that the remaining proteins occurred in the deeper lying fractions of the cuticle. The low molecular weight proteins did not appear to be extracted with the detergent in the later stages implying that these molecules too, may be different in the earlier stages and possibly more accessible to the extraction procedure. Thus it would appear that the only truly surface molecule present in the later stages (days 6-10) of the parasite was the 62 kDa and from days 1-5, the 15 kDa molecule. Thus, it would appear that those molecules which are known to be antigenic (Monroy, East, Dobson and Adams, 1989; Pritchard, Maizels, Behnke and Appleby, 1984; Pritchard and Carr, 1987), are present on the glycocalyx of the cuticle, whilst a wider spectrum of molecules which are possibly not recognised by the immune system are in the deeper lying portions of the cuticle. The recognition of the glycocalyx proteins may be due to increased exposure to the immune system as it has been shown (Adams, Monroy, East and Dobson, 1987; Pritchard, Maizels, Behnke and Appleby, 1984) that these molecules are present in parasite ES and so it is possible that they are actively shed into the medium and so readily accessible to the host immune response.

Parkhouse, Philipp and Ogilvie (1981) reported that molecules present on the surface of *T. spiralis* were immunogenic and of a restricted range, consisting of a 47 kDa glycoprotein and a 55 kDa protein. No cross reactivity was observed with other parasite species and it was demonstrated that the molecules were not secreted from the stichosome, which is known to secrete the majority of this parasites ES molecules, but were actively secreted from the cuticle. It was further demonstrated (Maizels, DeSavigny and Ogilvie, 1984; DeSavigny, 1975) that several antigenic components found in larval *Toxocara canis* ES were also present on the surface of the parasite. The larvae released 25% of surface bound radioactivity in less than 1 hour *in vitro*. This could not be attributed to the death of the parasites as they remained viable for many months.

Thus, the release of cuticular surface proteins must have some function. It has been proposed that they are released in order to divert the immune response away from damaging potentially more important structural molecules present on the cuticle. If these proteins were continually shed, then host antibodies that may have bound to the parasite surface would be sloughed off, thus preventing damage of the parasite cuticle through both the action of complement and effector cells. It was pertinent to note that no successful vaccine against nematode parasites has been devised using purified surface molecules (Philipp and Rumjaneck, 1984). However, it has also been shown that the infectivity of *T. spiralis* larvae was significantly reduced when they were incubated with an IgG_1 mouse monoclonal antibody directed against a 64 kDa surface protein (Ortega-Pierres, Chayen, Clark and Parkhouse, 1984). Therefore, it seems possible that if a strong enough antibody response could be mounted, this could overwhelm the secretory activity of the cuticle and thus facilitate the activation of both complement and the cellular arm of the immune response. Epitopes previously unseen by the immune system could be exposed and thus damage to the cuticle could occur on a greater scale and thus mediate the expulsion of the parasite.

CTAB stripping the parasites before radio-iodination revealed molecules which may be unavailable to a host undergoing a primary infection, but may be recognised by immune animals. From these investigations it was apparent that whilst the profile of days 6 and 7 did not appear to be altered considerably, the labelling was more intense but there were no extra bands nor missing bands in the samples. The profile of days 8-10 was quite different, not only were the 145 and 92 kDa molecules absent and others depleted (76, 62, 47 and 20 kDa), but extra molecules were labelled more strongly and additional bands were present. It appeared that the intense labelling of the 15 kDa band may have been due to the presence of several molecules, with varying susceptibilities to the extraction methods employed, with one being primarily associated with the B-ME fraction.

It would appear that those molecules which could be easily stripped using CTAB may not be as structurally important as those molecules lying in the deeper fragments of the cuticle beneath the glycocalyx. Stripping the parasite before labelling also allowed a greater proportion of the collagenous B-ME soluble proteins to be labelled (McKean and Pritchard, 1989).

Therefore, it seems likely that the glycocalyx proteins, which can be readily removed and are secreted from the cuticle, may be acting to circumvent the host immune response and direct it away from damaging more important structural molecules. It was interesting to note that CTAB stripping did not appear to damage the parasites. After 4 hours in the detergent at 37^{o}C they were still motile. It has been shown (Pritchard, Crawford, Behnke and Duce, 1985) that *T. spiralis* larvae were still infective after this treatment and there was no gross damage to the surface of *H. polygyrus*.

This mode of immune action has been proposed (McKean and Pritchard, 1989) to account for the expulsion of human hookworm, *N. americanus*. B-ME soluble proteins, collagens, were shown to be degraded by both bacterial collagenase and RMCP II. RMCP II is a mast cell protease specific for type IV

collagens and a mastocytosis is known to be a characteristic of gastrointestinal nematode expulsion (Woodbury et al, 1984). Thus, it was suggested that a wave of antibody could remove the parasite glycocalyx and then the action of RMCP II and the contents from other effector cells would then act on the collagenous molecules causing disruption of the parasite cuticle and ultimately, its death and expulsion from the host.

Overall, it appears that *H. polygyrus* does not possess the small collagenous molecules observed in the cuticle of *N. americanus* (Pritchard, McKean and Rogan, 1989). Although these molecules could be radio-labelled they were not immunogenic in naturally infected hosts, but an antibody response could be generated by injecting rabbits with adult worm homogenate. They suggested that this was because in the natural infection, the collagenous molecules were `masked' by other proteins on the epicuticle and thus were unavailable to the immune system.

Cox, Shamansky and Boisvenue (1989) also demonstrated the presence of two general classes of molecules on the surface of the cuticle. One class of proteins could be extracted with SDS whilst the other needed the presence of a disulphide reducing agent for solubilisation. The SDS soluble proteins of *H. contortus* L3 and L4 consist of relatively few major species with the pattern changing for each stage. The SDS fraction was not digestable with bacterial collagenase although the SDS/B-ME fraction was, this suggested that the collagenous molecules were in a deeper lying zone of the cuticle. The authors suggested that these molecules were derived from internal parts of the worm and not from the cuticle. Anti-sera prepared against the SDS soluble cuticles reacted strongly to the preparations but not at all on live worms. This suggested that even molecules in the SDS fraction were masked by proteins on the epicuticle and thus protected against the immune response.

4.3.4 Triton X 114 Extraction

Identification of hydrophobic proteins will, again, allow determination of the molecules that may be present in the inner rather than the outer layers of the parasite cuticle, as hydrophobic molecules may be more important structural proteins than the hydrophilic molecules. However, the extraction procedure was not entirely successful with very few labelled molecules being extracted in the detergent phase. This was probably because of the limited amounts of material available and the low stringency of the extraction procedure. It was also noted that very little material was extracted in the detergent phase and the molecules that were present were identical to those found in the aqueous phase. This implied that there were very few molecules in the nematode cuticle that were hydrophobic. However, it was shown earlier that the 62 kDa molecules may have some hydrophobic moieties as this was the predominant molecule extracted, alternatively, it may just be contaminating proteins.

It was interesting to note that that proportion of labelled material in the pellet increased with decreasing parasite age. This, again, is probably because this procedure is not very efficient at isolating molecules from the cuticle. Overall, this study suggested that the cuticle of *H. polygyrus* contained very few, if any, hydrophobic or amphiphilic molecules. It has been demonstrated in a number of protozoal parasites, *Leishmania major* and *Trypanosoma* spp. (Bouvier, Etges and Bordier, 1986) that the major surface proteins are anchored by a hydrophobic tail. However, as yet, this has not been demonstrated to be the case for nematode surface proteins. For instance, Devaney (1988) showed that the cuticular antigens of *Brugia malayi*, which were definitively localised to the epicuticle, did not have a hydrophobic tail. This is not totally surprising because proteins with hydrophobic tails are usually thought to be associated with lipid bilayer membranes (Bouvier, Etges and Bordier, 1986) and not with the properties of the nematode cuticle, which at one time was thought to be a

totally inert structure. They also demonstrated that the surface glycoproteins of *L. major* were fixed into the membrane with an easily releasable hydrophobic lipid anchor, whilst it is known that the nematode cuticle is an acellular matrix containing collagens and other structural proteins.

4.3.5 Quantitative Analysis of Labelling Procedures

The quantitative differences in incorporation of the label between L3 and days 1 and 2 were quite remarkable. In L3, a very small portion of the counts could be CTAB stripped, but by day 2, this was nearly 90% of the total labelled material. In the cuticle preparations this was equivalent to those molecules which were extracted by SDS. By day 3 the proportion of molecules in the CTAB supernatant and/or detergent phase has started to decline and by days 4 and 5, the CTAB supernatant contains a minimal number of counts. However, the majority of the label is mainly in the homogenate, implying that the cuticular molecules were altering in some way, perhaps as a result of a change in the charge of the molecules, making them more cationic and thus less susceptible to the action of the cationic detergent, CTAB. The percentage of label incorporated in each fraction and preparation changed very little between day 6 and adult although the amount removed by CTAB increased with the age of the parasite, which again suggested that either the charge property of the molecule was altering or the numbers of removable molecules has increased.

These changes, again, appear to be associated with the developmental stage of the parasite. The exsheathing of the parasite from an L3 to day 1 results in one major change followed by another when the worm moults from L3 to L4 on day 4 and a final moult on day 6 when the parasite develops from a L4 to an adult. Other changes may also be associated with changing environment of the parasite as it changes from the free-living L3 to a tissue-dwelling L4 and then finally to a lumen residing adult. The sexual maturity of the parasite may also affect the expression of molecules on the parasite surface

as chemoattractants are released, mating occurs and egg production begins (Ortega-Pierres, Clark and Parkhouse, 1985; Bone and Bottjer, 1985).

From these studies it was apparent that the molecules in the deeper lying zones of the nematode cuticle did not vary considerably. It has been suggested (Philipp and Rumjaneck, 1984; Lee, 1977) that cuticular proteins are synthesised in the hypodermis and translocated to the cuticular surface via pores; they are then shed into the environment either by being displaced by new molecules or by cleavage by surface proteases. However, there is also some evidence that synthesis may take place in the cortical layer as RNA has been found in this layer in ascarids (Anya, 1966) and transmembraneous particles have been observed in the epicuticle of *N. brasiliensis* (Lee and Bonner, 1982).

The demonstration of sex specific molecules on the surface of this parasite could explain why the male:female ratio alters in favour of the female worm in an immune response to the parasite. Recognition of male proteins in preference to female molecules would result in the elimination of the parasite and/or disruption of chemoattraction and mating. This may also explain why reduced worm fecundity occurs in immune animals if the molecules recognised are associated with the female parasite and reproductive organs (Adams, East, Monroy and Dobson, 1988)

Stage specific molecules have been widely reported on the surfaces of other parasitic nematodes such as *A. suum* (Kennedy and Qureshi, 1986), *H. contortus* (Cox, Shamansky and Boisvenue, 1989), *T. spiralis* (Philipp, Parkhouse and Ogilvie, 1980), *T. canis* (Maizels, DeSavigny and Ogilvie, 1984), *N. brasiliensis* (Maizels, Meghji and Ogilvie, 1983), *N. americanus* (Pritchard, Behnke, Carr and Wells, 1986; Carr and Pritchard, 1987) and *O. gibsoni* (Forsyth, Copeman and Mitchell, 1984) as well as on the surface of *H. polygyrus* (Pritchard, Maizels, Behnke and Appleby, 1984; Adams, East, Monroy, Washington and Dobson, 1987). The function of these stage specific proteins is not fully understood. A number of suggestions have been made for

the possible mode of action of the wide variety of molecules demonstrated to be present. These include enzymes required for feeding, exsheathing and penetrating the host, chemoattractants and their receptors, `antigenic disguise', and modulators of the host immune system. Immunity to *H. polygyrus* is known to be directed at the larval stages rather than at the adult parasite (Behnke and Parish, 1979b) and the adult worm is thought to be capable of actively suppressing the hosts immune system (Behnke, Hannah and Pritchard, 1983) to both homologous and heterologous challenge. Therefore, identification of stage specific molecules may assist in the isolation of those molecules responsible for either stimulating or suppressing the host immune response to this parasite.

In conclusion, it can be seen that *H. polygyrus* expresses a number of stage specific surface molecules. The expression of these increases in variety as the parasite develops and matures. A limited number of these were removed using the cationic detergent CTAB, which suggested that only a limited fraction of the molecules present could be accessible to the immune system. If the outer layer was removed, more molecules were revealed in the lower layers of the cuticle, again, implying a parasite protective role for the epicuticular proteins. Hydrophobic molecules did not appear to be crucial for the maintenance of the structural integrity of the cuticle. In order to ascertain which molecules were responsible for eliciting a functional immune response against the parasite, a number of the preparations were precipitated using a range of immune sera developed in Chapters 5 and 6, the results are discussed in Chapter 7. The presence of collagenous molecules in the surface of this parasite would need to be confirmed using bacterial collagenase and/or RMCP II on the various preparations of the various parasite stages, thus revealing which molecules are most structurally important.

CHAPTER FIVE

THE INFLUENCE OF MOUSE STRAIN ON IMMUNOLOGICAL RESPONSE TO A PRIMARY INFECTION WITH *HELIGMOSOMOIDES POLYGYRUS*

5.1 INTRODUCTION

A primary infection of mice with *Heligmosomoides polygyrus* results in a chronic infection of 8-10 month duration (Robinson, Wahid, Behnke and Gilbert, 1989). However, different strains of the mouse host show varying degrees of resistance to the parasite. For instance, some strains such as SJL and SWR are classified as high responders and expel the worm after about 6 weeks whereas others such as BALB/c, NIH, B10G and DBA are intermediate responders expelling the worm between 10 and 20 weeks after infection, whilst others such as CBA, C_3H and C57BL/10 are low responders, taking up to 35 weeks to expel the parasite. It has been proposed that the time taken for low responder strains to expel the parasite probably reflects the natural life span of the parasite within the host rather than an immune response to the parasite (Behnke, Williams, Hannah and Pritchard, 1987).

The ability of these various strains of the mouse host to expel the parasite appears to be associated with the phenotype of the major histocompatibility complex (MHC). Observations by Behnke and Robinson (1985) suggested that both H-2 and non-H-2 genes were responsible for immunity to both a primary and challenge infection. It appears that q and s haplotypes are associated with resistance whilst k and b haplotypes are associated with susceptibility with the d haplotype representing a range of responses between. However, it seems that not only H-2 genes are responsible for this phenomenon as mice of the same haplotype but with different background genes show a range of response to the parasite.

Similarly, studies by Enriquez, Brooks, David and Wassom (1988) have demonstrated an H-2 control of the response to this parasite. Strains of mice expressing the k-allele were much more susceptible to infection than those expressing the f, q or s-allele. Again, the background genes also seemed to modify the immune reaction to the parasite as mice with the same haplotype but different backgrounds demonstrated a variety of reactions to the parasite.

Wahid and Behnke (unpublished observations) demonstrated, using F1 mice, that the different haplotypes could influence each other. For instance, crossing a high responder and a low responder strain results in offspring (q x b) with intermediate responsiveness and that crossing two high responders of different haplotype SWR x SJL (q x s) gives offspring whose response to the parasite is greater than either parent. This implies that the genes are co-dominant and can influence the overall phenotype of the mouse host.

Much work on the responsiveness of strains to the infection has involved studying worm burdens over a time period or the ability of the host to resist a challenge infection. There have been many studies analysing the various immunological responses to the parasite and some of these have related this to the strain of the mouse host. Few studies have tried to link these responses to the haplotype of the mouse or have used a primary infection in order to investigate them.

For a number of reasons female mice were employed throughout all of the experiments described. Firstly, female mice are thought to be more resistant to *H. polygyrus* infection than male mice (Dobson and Owen, 1978), possibly as a result of suppression of lymphocyte activity by the male sex hormone, testosterone or enhancement of the reticuloendothelial system by the female sex hormone, oestrogen. Robinson, Wahid, Behnke and Gilbert (1989) demonstrated that low intensity inoculations in male BALB/c mice resulted in infections which lasted for twice as long as those seen in female mice exposed to similar doses, but which were similar in duration to high intensity infections in female mice. Thus, the use of female mice to compare expulsion of the parasite from a primary infection between different strains of mice allowed this phenomenon to be investigated over a shorter time course and with greater separation between the strains than would be possible with male mice. Secondly, female mice are not as aggressive or as susceptible to stress as male mice. Indeed, male SJL mice are so aggressive that they need to be housed individually or they will kill each other within 4 months and in our animal house

this was not feasible. Finally, female mice were more readily available from both the departmental animal house and Harlan-Olac (Oxon).

5.2 PRELIMINARY INVESTIGATION

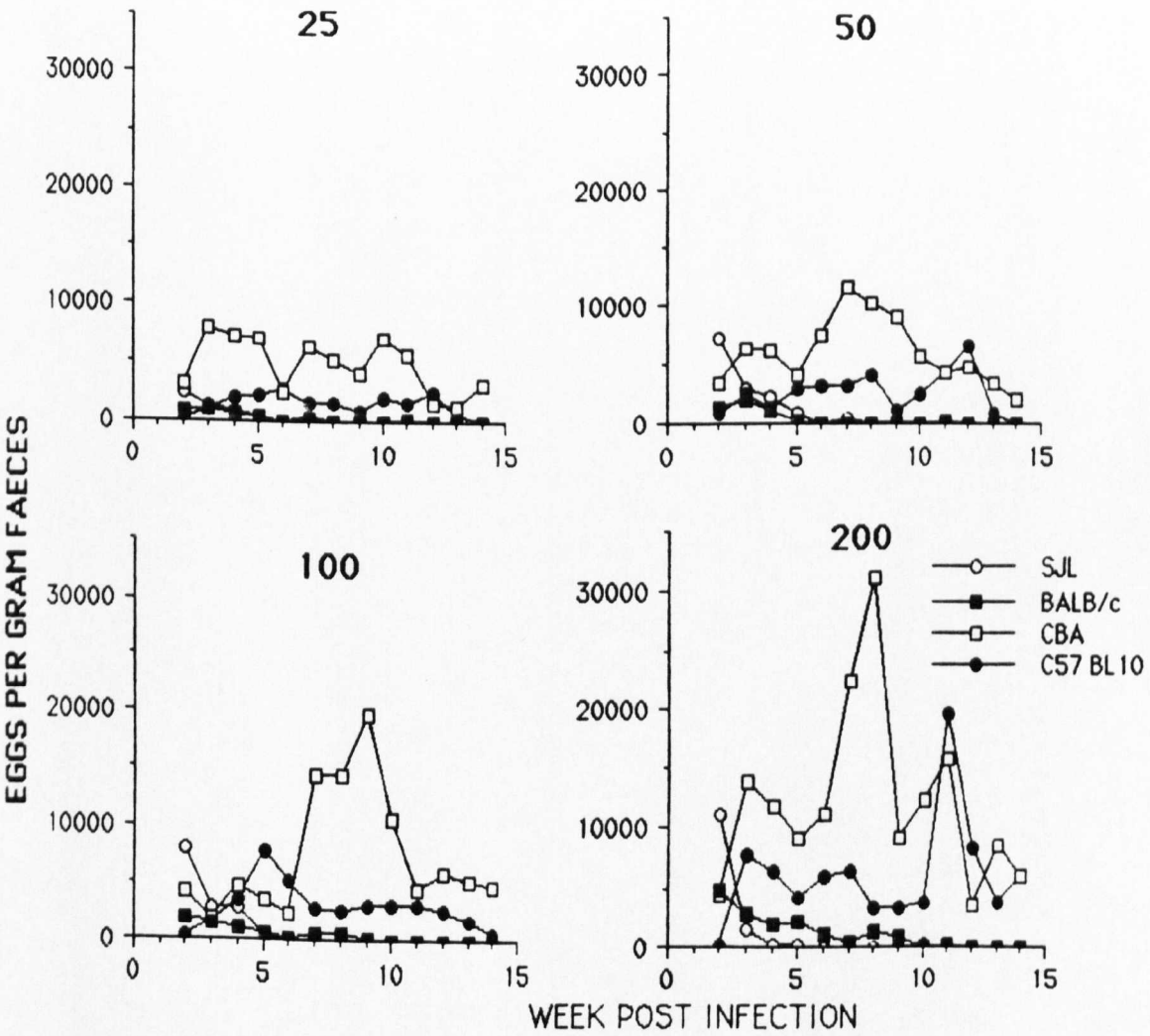
A preliminary investigation was necessary in order to select the strains to be used and the infection intensities required. Four strains of female mice of known responsiveness to *H. polygyrus* were exposed to a range of infection levels. The strains employed were SJL, C57BL/10, CBA and BALB/c and the levels of infection used were 25, 50, 100 and 200 L3. Egg counts were performed weekly for 15 weeks and at the end of this period the mice were sacrificed and the worm burdens assessed using the modified Baermann technique.

5.2.1 RESULTS

As can be seen from Fig. 5.1 the different strains and different infection intensities produced a varying egg count profile over this time period. CBA and C57BL/10 produced the greatest number of eggs at all infection intensities followed by BALB/c and then SJL. It was also noted that SJL stopped egg production between weeks 5 and 7, BALB/c between weeks 10 and 15 whilst CBA and C57BL/10 did not cease egg production for the duration of the experiment.

Figure 5.1 Faecal Egg Counts

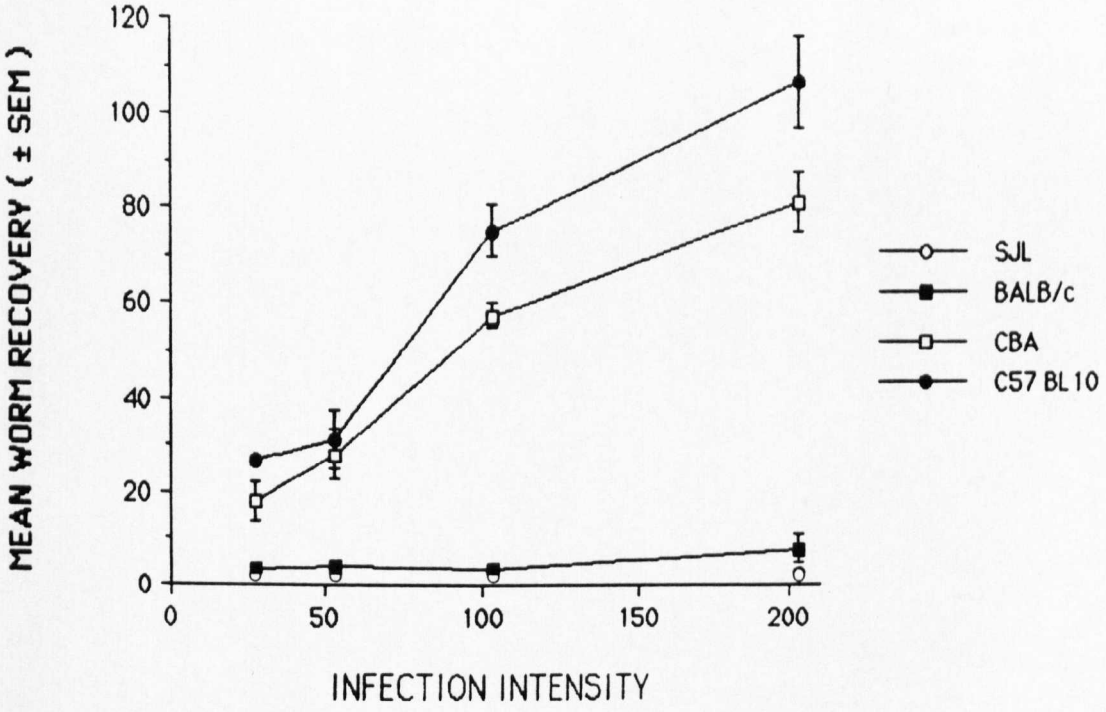
Faecal egg counts were performed weekly by standard zinc sulphate flotation for four strains of mice infected with 25, 50, 100 or 200 *H. polygyrus* infective larvae.


25
50
100
200
30000
20000
10000
0
0
5
10
15
EGGS PER GRAM FAECES
WEEK POST INFECTION
SJL
BALB/c
CBA
C57 BL10

When the worm burdens were assessed (Fig. 5.2) it was noted that SJL and BALB/c had expelled all of their worms at all infection intensities whereas CBA and C57BL/10 still harboured a significant worm burden, the level of which was proportional to the original intensity of infection.

Figure 5.2 Worm Burdens

The worm burdens of four strains of mice given a primary infection with 25, 50, 100 or 200 infective *H. polygyrus* larvae were assessed 15 weeks post infection using the modified Baermann technique. The mean and standard error for each time point was calculated.


MEAN WORM RECOVERY (± SEM)
120
100
80
60
40
20
0
0
50
100
150
200
INFECTION INTENSITY
SJL
BALB/c
CBA
C57 BL 10

5.2.2 DISCUSSION

From the results of this preliminary experiment it can be seen that overall, at all infection intensities CBA and C57BL/10 are low responders, BALB/c are medium responders and that SJL are high responders to this parasite. The level of infection which seemed to give the best separation of the strains in terms of weekly egg counts and expulsion of the parasite was 200 L3. The strains of mice which seemed to best represent high, medium and low responders and gave the greatest range of responses were SJL, BALB/c and CBA, respectively and so these were used in all subsequent experiments.

5.3 PRIMARY INFECTION

As a result of the initial experiment previously described it was decided to use SJL, a high responder strain, BALB/c, and CBA a low responder strain at an infection intensity of 200 L3. This was shown to give the greatest separation of the strains in terms of egg counts and worm expulsion and so it was thought that they would best represent a range of immune responses to the parasite.

The aim of the experiment was to study a number of immunological parameters and to try to correlate these with the ability of the host strain to expel the parasite. Each strain of mouse was killed before, and during or near after, expulsion of the parasite as assessed by the fall in egg counts. Egg counts were monitored weekly and when they had dropped to zero for two concurrent weeks, a group of that strain was sacrificed immediately. Behnke and Parish (1979b) and Sitepu and Dobson (1982) demonstrated that faecal egg counts were related to the worm burden of the host and so it was estimated that if the egg counts were zero for two consecutive weeks then the parasite had been expelled. The experimental design was briefly as follows, groups of six mice were pre-bled and blood smears and white blood cell counts carried out. They were then orally dosed with 200 infective larvae. One group of each strain of mice was killed three weeks after infection in order to assess the infectivity of the larvae. A group was then killed from each strain before and after expulsion, for SJL this was on weeks 4 and 6, for BALB/c on weeks 6 and 13 and CBA on weeks 6, 13 and 35. White blood cell counts, blood smears and sera were taken every fortnight until the experiment terminated and at each autopsy a number of immunological parameters were investigated. These included, the cellularity of the secondary lymphoid organs, analysis of the cell population of these organs and their response to mitogens, enumeration of mast cells in the gut and analysis of the humoral response to parasite antigens by enzyme linked immuno-sorbent assay (ELISA) and immuno blotting.

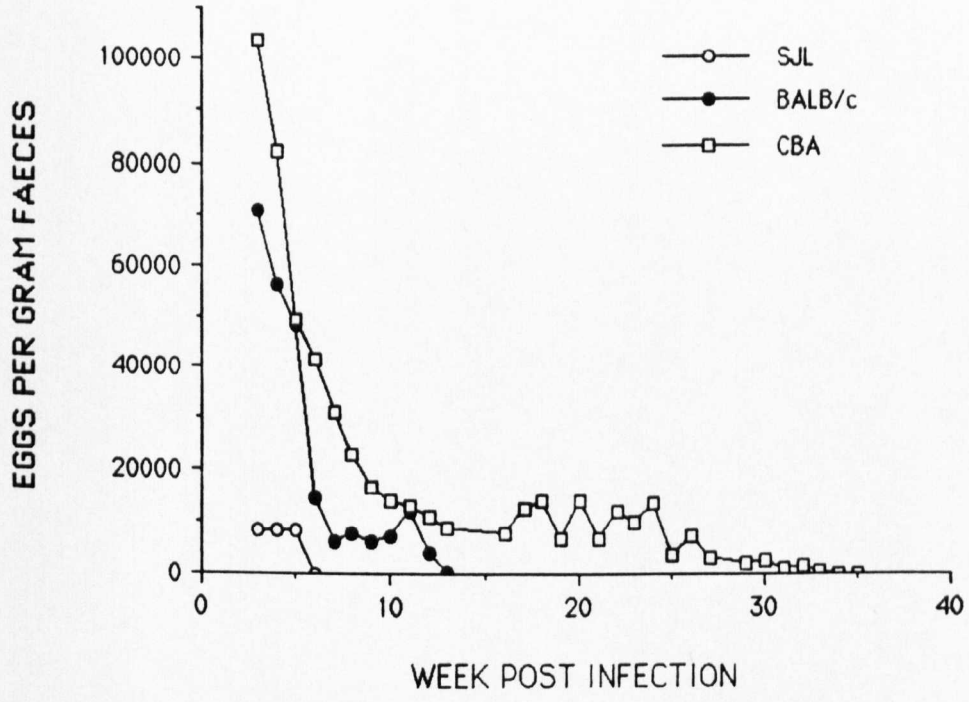
5.4 RESULTS

5.4.1 Faecal Egg Counts

Fig. 5.3 shows the egg counts in each strain of mouse as the infection progressed. The initial faecal egg output of SJL was about one tenth of that of BALB/c and CBA and rapidly declined to zero by week 6. BALB/c and CBA showed the same general pattern of decline but this was more rapid in BALB/c reaching zero by 13 weeks rather than 35 weeks as with CBA.

Figure 5.3 Faecal Egg Counts

Faecal egg counts were performed weekly by standard zinc sulphate flotation in SJL, BALB/c and CBA mice infected with 200 *H. polygyrus* larvae. This continued until the worms were expelled from the host.


EGGS PER GRAM FAECES
100000
80000
60000
40000
20000
0
0
10
20
30
40
WEEK POST INFECTION
SJL
BALB/c
CBA

5.4.2 Worm Burdens

Fig. 5.4 represents the worm burdens at each autopsy. At week three the intensity of infection was approximately equal for all strains. However, by 6 weeks SJL have lost 91.9% of their worms whereas CBA and BALB/c have maintained their initial infection level. By week 13 BALB/c have only 13.7% of their initial parasite burden remaining whilst CBA still have 92.9% but by week 35 this has declined to 15.3%.

Figure 5.4 Worm Burdens

The parasite burdens of SJL, BALB/c and CBA mice infected with 200 *H. polygyrus* larvae was assessed using the modified Baermann technique at various time points post infection. The mean and standard error for each time point was calculated.

SJL

Week 3 vs 4: NS 3 vs 6: $p < 0.005$ 4 vs 6: $p < 0.005$

BALB/c

Week 3 vs 6: NS 3 vs 13: $p < 0.005$ 6 vs 13: $p < 0.05$

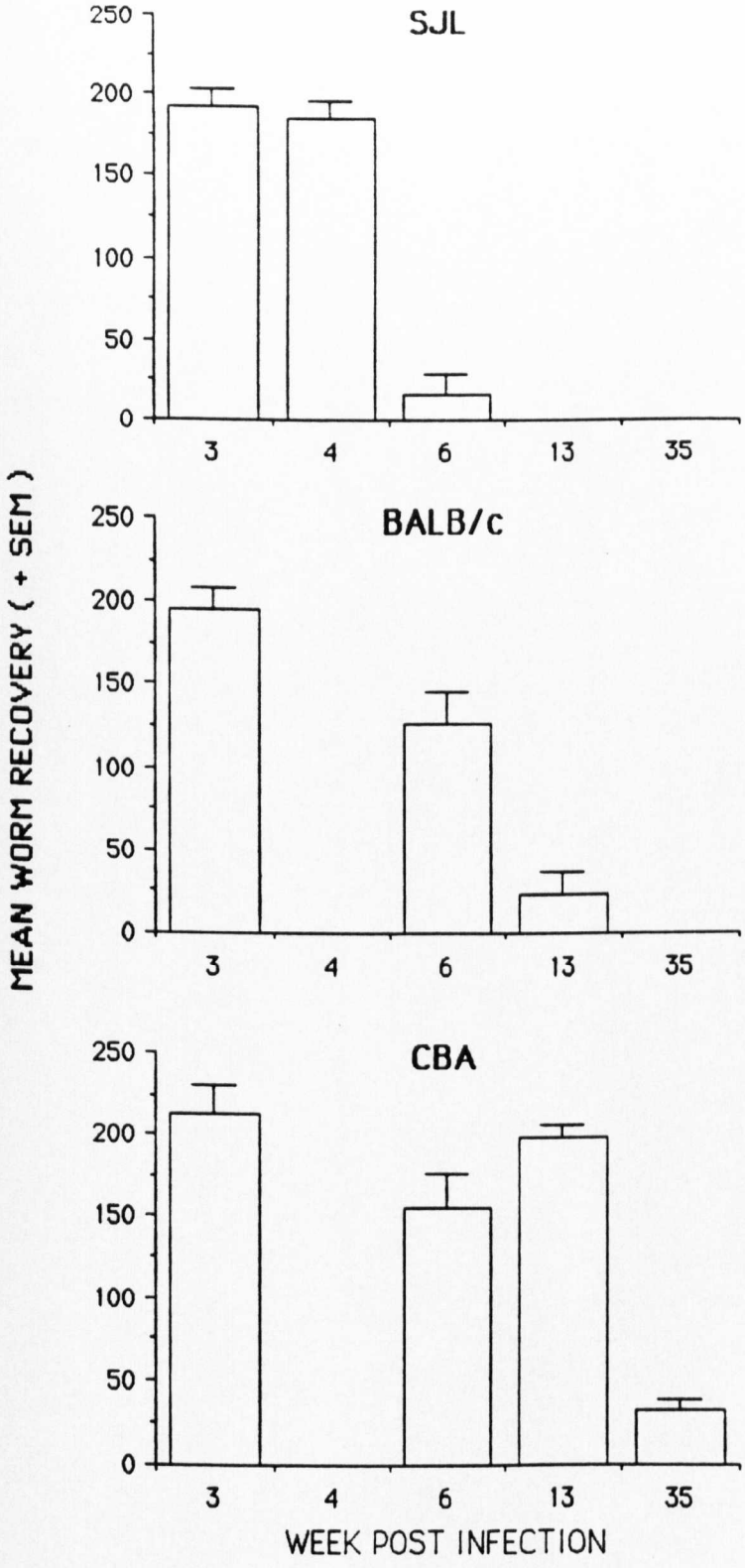
CBA

Week 3 vs 6: NS 3 vs 13: NS 3 vs 35: $p < 0.005$

SJL6 vs BALB/c6: $p < 0.05$ SJL6 vs CBA6: $p < 0.05$

CBA6 vs BALB/c6: NS CBA13 vs BALB/c13: $p < 0.005$

N.B. NS (not significant)


SJL
BALB/c
CBA
250
200
150
100
50
0
3
4
6
13
35
MEAN WORM RECOVERY (+ SEM)
WEEK POST INFECTION

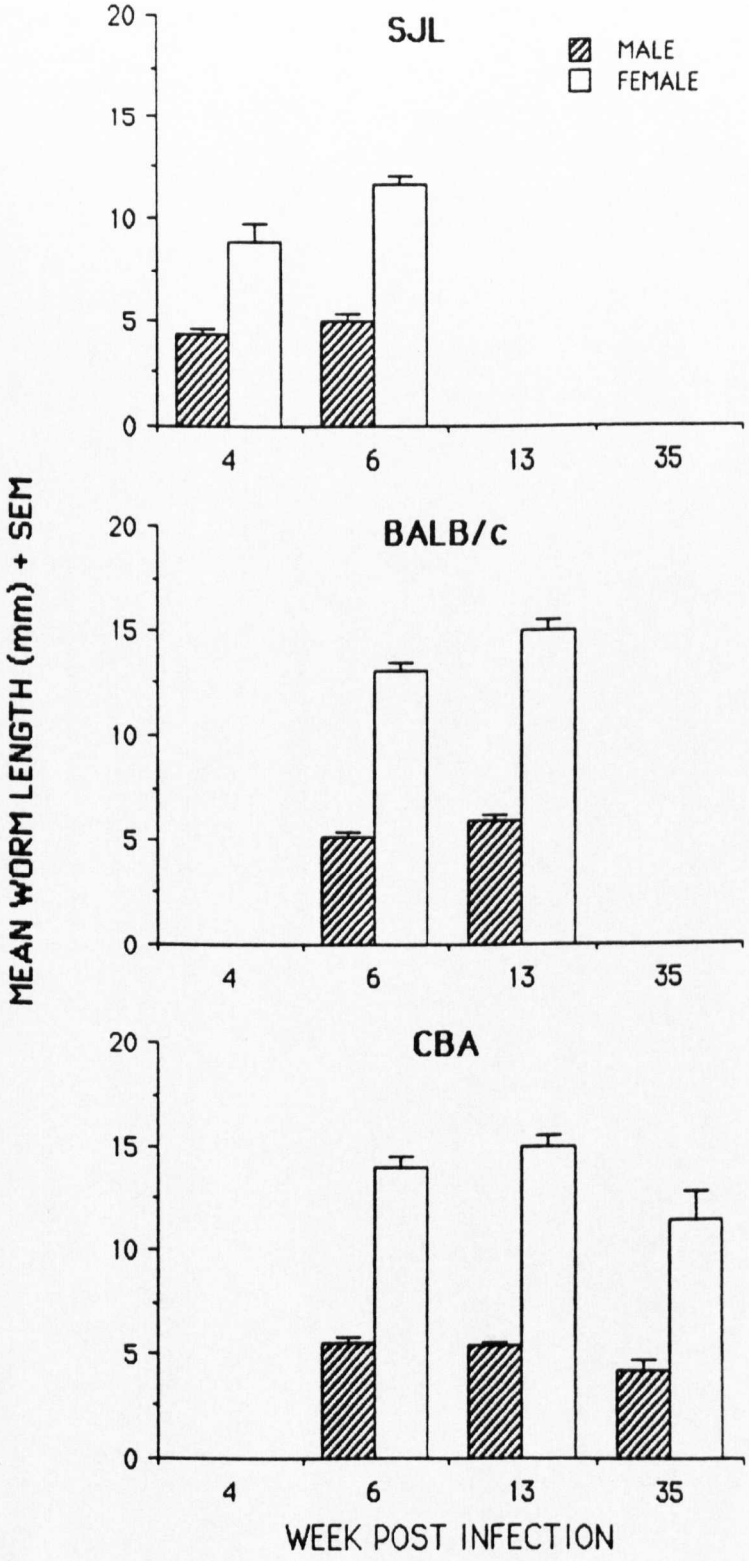
5.4.3 Worm Lengths

When the size of the parasites at each autopsy were assessed it was noted from Fig. 5.5 that the average length of the remaining parasites at each autopsy, did not appear to decrease markedly although there was a suggestion of an increase as the infection progressed.

Figure 5.5 Parasite Length

The length of parasites obtained from SJL, BALB/c and CBA mice at various time points post infection was assessed by drawing the parasites with the aid of a camera lucida and the length of the drawings was measured with a bit-pad digitiser linked to an Apple PC. The mean and standard error for each time point was calculated.

SJL4 vs 6: $p < 0.05$	BALB/c6 vs 13: $p < 0.05$
CBA6 vs 35: NS	CBA13 vs 35: $p < 0.05$
SJL6 vs BALB/c6: NS	SJL6 vs CBA6: $p < 0.05$


SJL
MALE
FEMALE
BALB/c
CBA
MEAN WORM LENGTH (mm) + SEM
WEEK POST INFECTION
0
5
10
15
20
4
6
13
35

5.4.4 White Blood Cell Counts

The cellular response of the host to the parasite was assessed on a number of levels. Firstly, the cellularity of the peripheral blood was calculated by using the total white blood cell (WBC) counts whilst the change in proportion and numbers of each cell population was examined by doing differential cell counts on the blood smears.

The peripheral WBC response showed a sharp rise in the total number of cells (Fig. 5.6) which appeared to be temporally related to the fall in egg counts. SJL responded more quickly and to a greater degree than the other two strains.

Figure 5.6 White Blood Cell Counts

The numbers of white blood cells present in the blood of infected SJL, BALB/c and CBA mice was measured every fortnight. The mean and standard error of the number of cells present per ul of blood was calculated.

SJL

Week 0 vs 4: NS 0 vs 6: $p < 0.05$ 4 vs 6: NS

BALB/c

Week 0 vs 6: NS 0 vs 13: NS 6 vs 13: NS

CBA

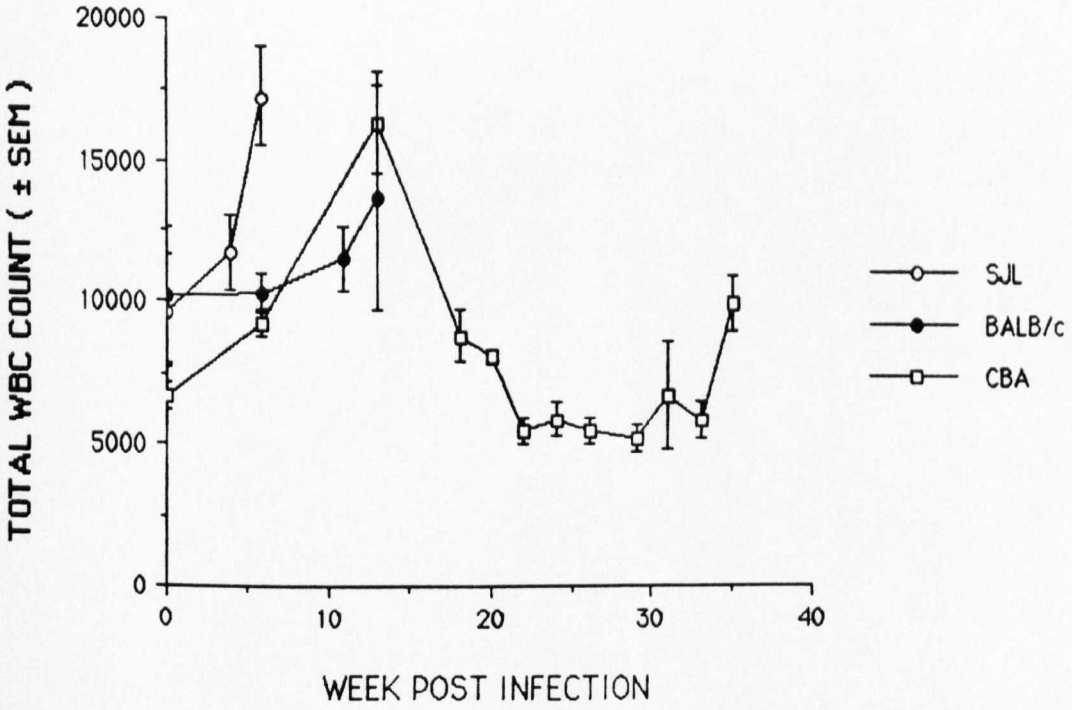
Week 0 vs 6: $p < 0.005$ 0 vs 13: $p < 0.05$

Week 0 vs 35: $p < 0.05$ 13 vs 35: $p < 0.05$

SJL6 vs BALB/c6: $p < 0.05$ SJL6 vs CBA6: $p < 0.05$

CBA6 vs BALB/c6: NS CBA13 vs BALB/c13: NS

N.B. NS (not significant)


TOTAL WBC COUNT (± SEM)
20000
15000
10000
5000
0
0
10
20
30
40
WEEK POST INFECTION
SJL
BALB/c
CBA

5.4.5 Differential Cell Counts

When the differential cell counts were performed at each time point (Fig. 5.7) it was noted that the percentage of eosinophils did not alter markedly. The percentage of neutrophils did not appear to change in SJL or CBA but rose in BALB/c. The percentage of monocytes dropped in all strains and there was a substantial rise in the percentage of lymphocytes in SJL and CBA but not in BALB/c.

Figure 5.7 Differential Cell Percentages

The percentages of lymphocytes, monocytes, eosinophils and neutrophils was calculated fortnightly from Giemsa stained blood smears taken from infected SJL, BALB/c and CBA. The mean and the standard error was calculated.

Lymphocytes

SJL

Week 0 vs 4: $p < 0.05$ 0 vs 6: $p < 0.05$ 4 vs 6: NS

BALB/c

Week 0 vs 6: NS 0 vs 13: $p < 0.05$ 6 vs 13: $p < 0.05$

CBA

Week 0 vs 6: NS 0 vs 13: NS

Week 0 vs 35: $p < 0.05$ 13 vs 35: $p < 0.05$

SJL6 vs BALB/c6: $p < 0.005$ SJL6 vs CBA6: $p < 0.005$

CBA6 vs BALB/c6: NS CBA13 vs BALB/c13: NS

Eosinophils

SJL

Week 0 vs 4: NS 0 vs 6: NS 4 vs 6: NS

BALB/c

Week 0 vs 6: $p < 0.05$ 0 vs 13: NS 6 vs 13: NS

CBA

Week 0 vs 6: NS 0 vs 13: NS

Week 0 vs 35: NS 13 vs 35: NS

SJL6 vs BALB/c6: NS SJL6 vs CBA6: NS

CBA6 vs BALB/c6: NS CBA13 vs BALB/c13: NS

N.B. NS (not significant)

Monocytes

SJL

Week 0 vs 4: $p < 0.05$ 0 vs 6: $p < 0.05$ 4 vs 6: NS

BALB/c

Week 0 vs 6: NS 0 vs 13: $p < 0.05$ 6 vs 13: NS

CBA

Week 0 vs 6: NS 0 vs 13: $p < 0.005$

Week 0 vs 35: $p < 0.005$ 13 vs 35: NS

SJL6 vs BALB/c6: NS SJL6 vs CBA6: $p < 0.05$

CBA6 vs BALB/c6: NS CBA13 vs BALB/c13: NS

Neutrophils

SJL

Week 0 vs 4: NS 0 vs 6: NS 4 vs 6: NS

BALB/c

Week 0 vs 6: NS 0 vs 13: $p < 0.005$ 6 vs 13: $p < 0.05$

CBA

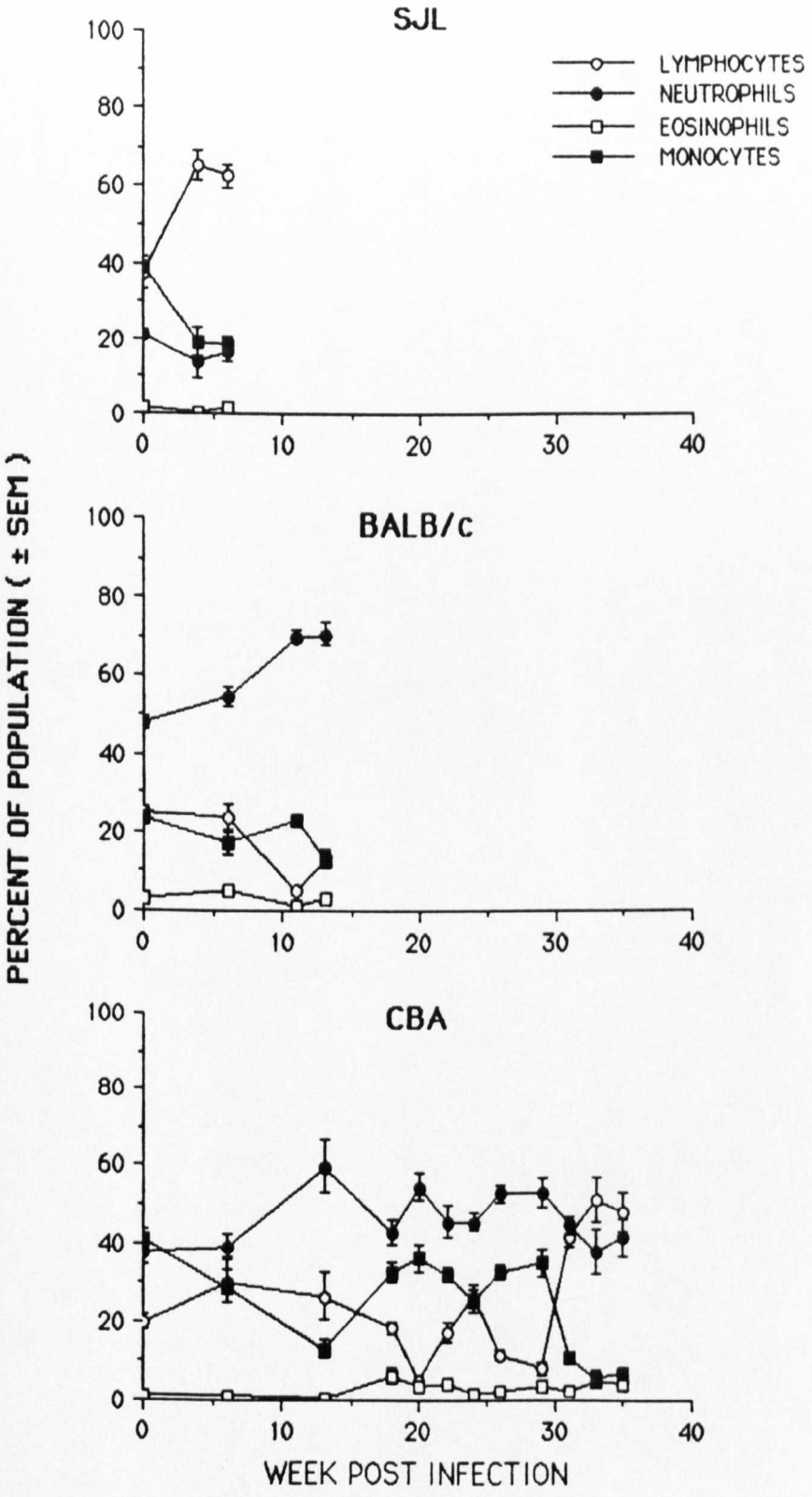
Week 0 vs 6: NS 0 vs 13: $p < 0.05$

Week 0 vs 35: NS 13 vs 35: NS

SJL6 vs BALB/c6: $p < 0.005$ SJL6 vs CBA6: $p < 0.05$

CBA6 vs BALB/c6: NS CBA13 vs BALB/c13: $p < 0.05$

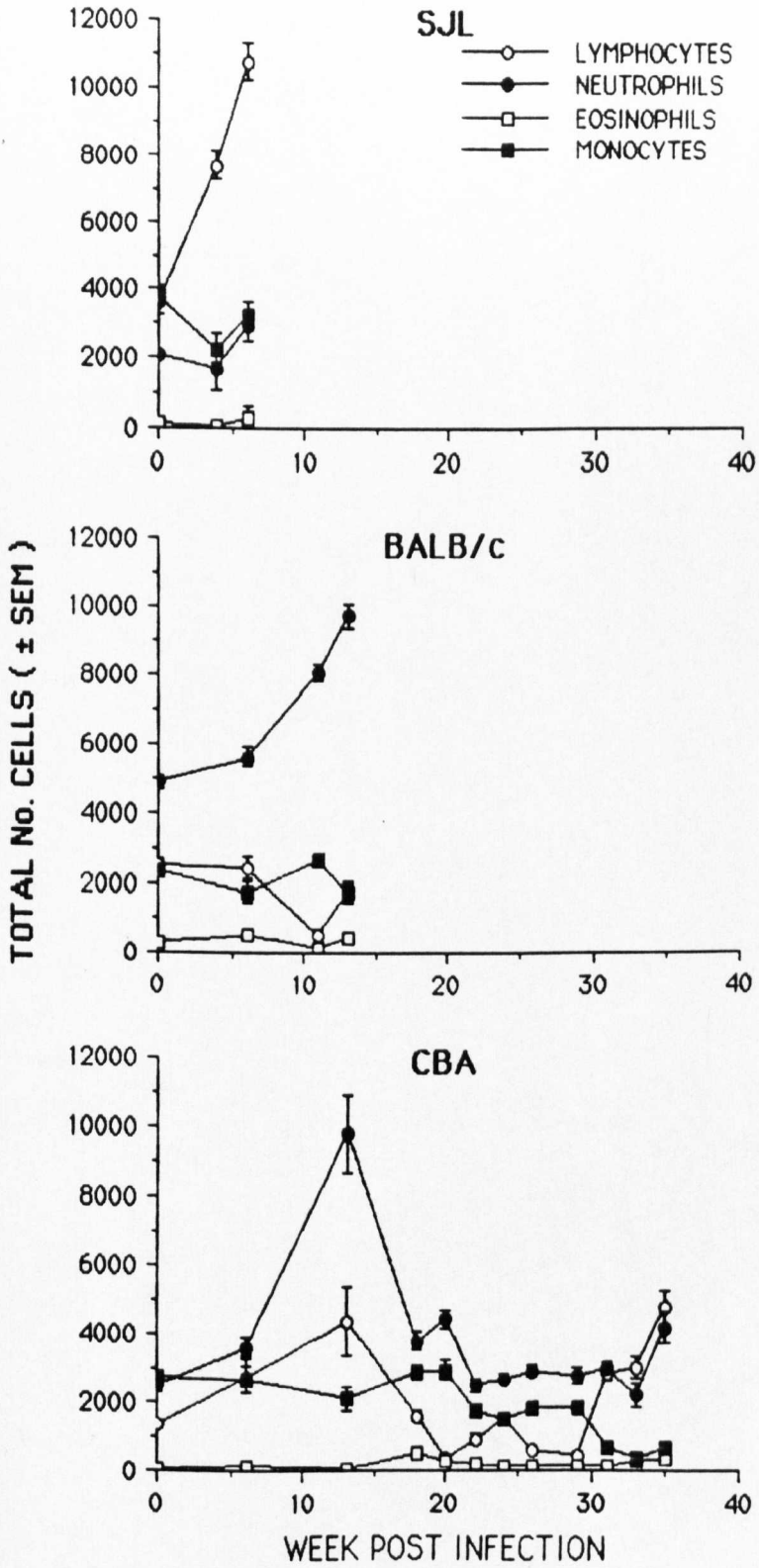
N.B. NS (not significant


SJL
LYMPHOCYTES
NEUTROPHILS
EOSINOPHILS
MONOCYTES
BALB/c
CBA
PERCENT OF POPULATION (± SEM)
WEEK POST INFECTION
0
20
40
60
80
100
10
30

When the total numbers of the cell types was calculated (Fig. 5.8) the trends became more apparent. The total number of eosinophils rose very slightly whilst the numbers of monocytes dropped insignificantly in all of the strains examined. The numbers of neutrophils rose in all strains but was most obvious in BALB/c, whereas lymphocytes were the predominant cell population in SJL, rising only slightly in BALB/c and CBA.

Figure 5.8 Total Differential Cell Numbers

The total numbers of lymphocytes, monocytes, eosinophils and neutrophils were calculated for each strain of infected mouse by using the total white blood cell counts and the percentages. The mean and standard error for each was calculated.


SJL
LYMPHOCYTES
NEUTROPHILS
EOSINOPHILS
MONOCYTES
BALB/c
CBA
TOTAL No. CELLS (± SEM)
WEEK POST INFECTION
12000
10000
8000
6000
4000
2000
0
0
10
20
30
40

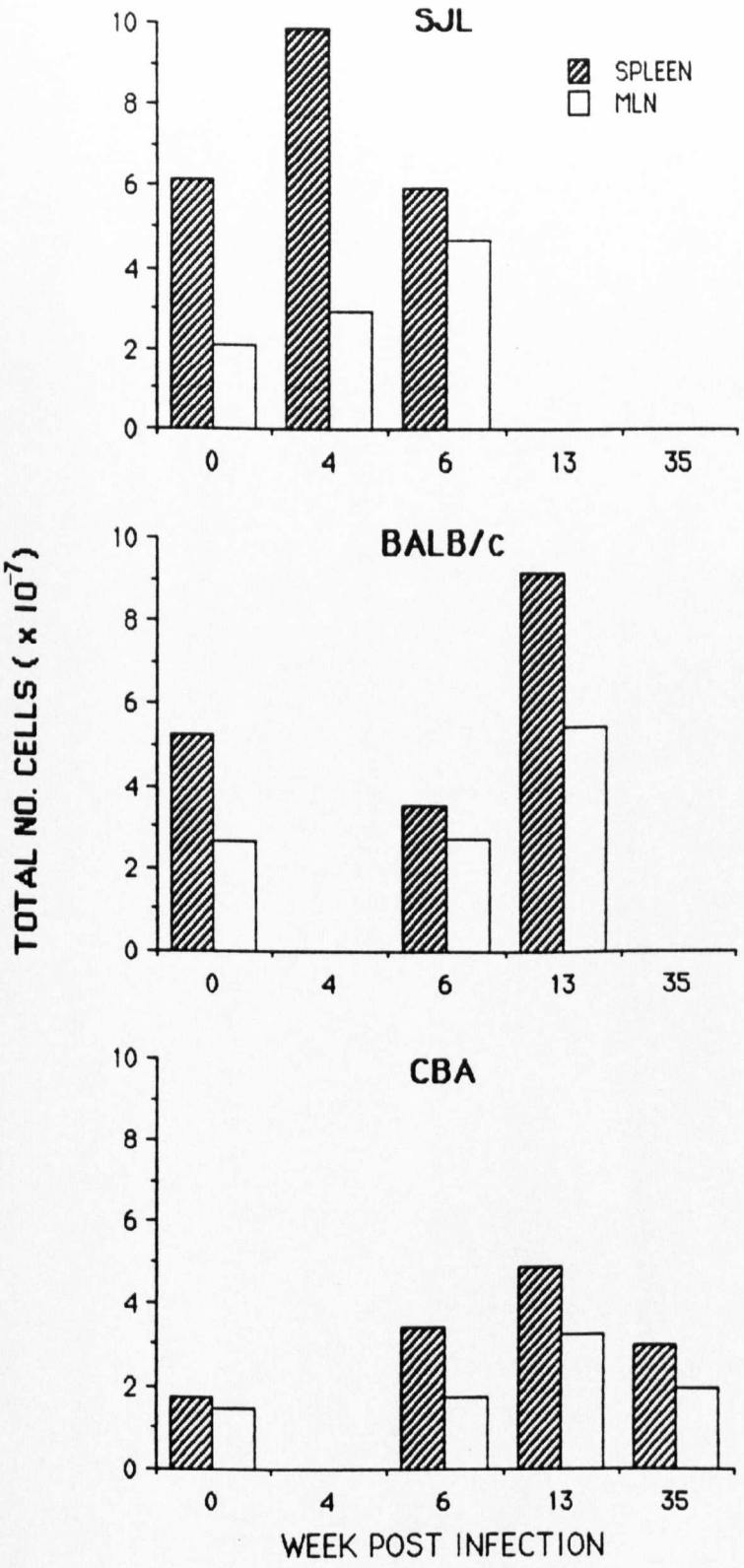
5.4.6 Cellular Response of the Secondary Lymphoid Organs

The changes occurring in the secondary lymphoid organs were also assessed. The organs of primary interest in this study were the mesenteric lymph nodes (MLN), those nodes draining the site of the infection, the gut, and the spleen which is thought to act as a `back up' to the MLN and help in processing parasite antigens and toxins. The importance of the various cell populations within these organs was investigated by enumerating the T and B cells in each organ. The responsiveness of the cells within these organs was appraised by measuring their blastogenic index and their ability to respond to the mitogens concanavalin A (Con A) and lipopolysaccharide (LPS). Unfortunately, it was necessary to use pooled cells from both the spleen and MLN for each group of animals studied as if the organs were prepared individually, the cells from them died before they could be used. Thus, no statistical analysis could be performed on this data.

The total number of viable cells was assessed by their ability to hydrolyse fluorescein diacetate (FDA) to free fluorescein. It was decided to assess the total number of viable cells rather than the wet weight of the organ as an increase in weight could be the result of oedema or increased production or breakdown of red blood cells rather than cellular proliferation or infiltration of the organ (Losson, Lloyd and Soulsby, 1985). From Fig. 5.9 it can be seen that the total number of cells in the spleen increased in response to infection although in SJL and CBA it appeared to fall before the parasite was expelled. In the mesenteric lymph nodes, the trend was more clear cut, the size of the organs increased until the parasite was expelled. Again, SJL responded quicker and to a greater degree than BALB/c which in turn was more responsive than CBA.

Figure 5.9 Total Cellularity of the Secondary Lymphoid Organs

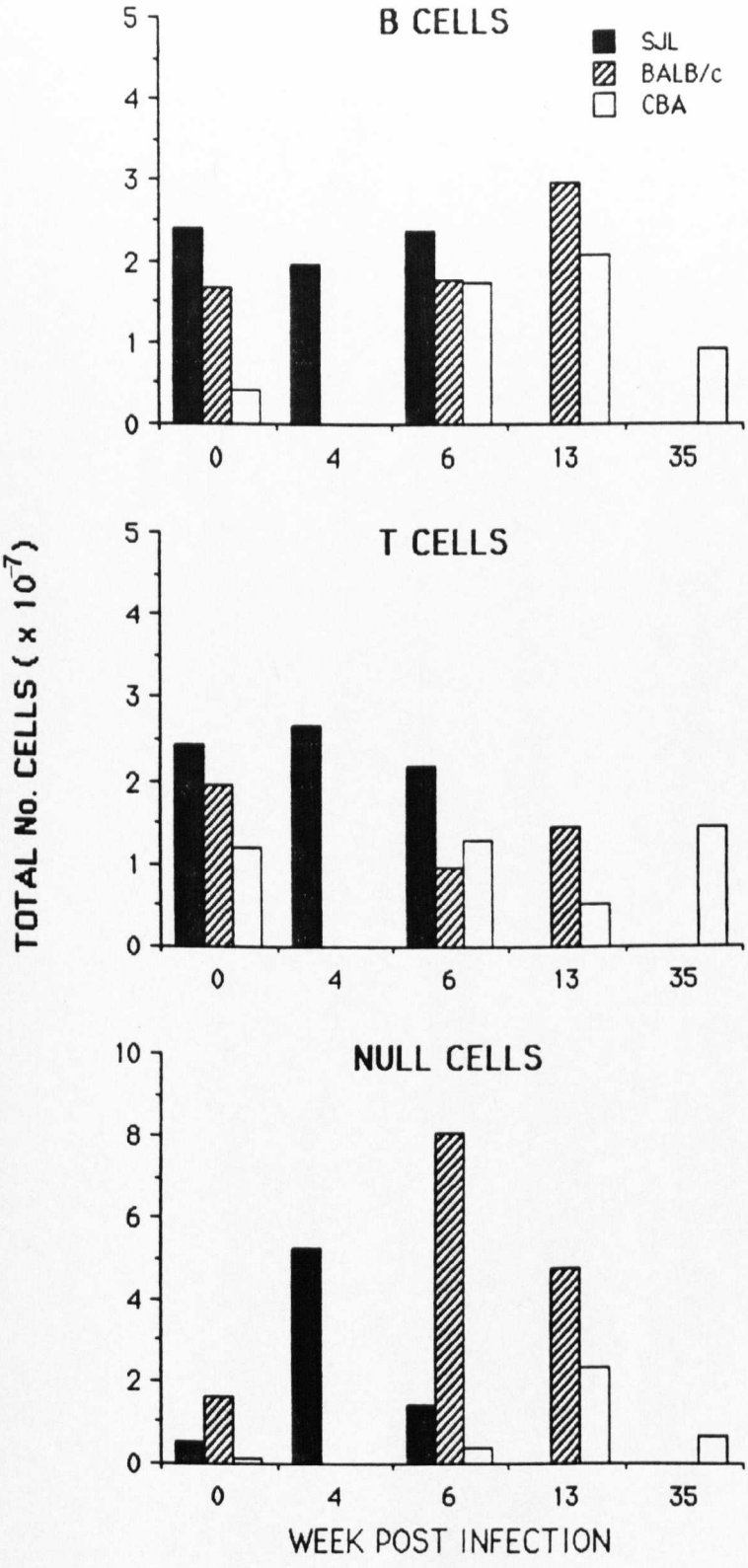
The total number of viable cells present in the spleen and the MLN of infected SJL, BALB/c and CBA mice was calculated at each autopsy by assessing their ability to convert fluorescein diacetate to free flourescein. Pooled cells were used in each group and so the mean number of cells per mouse is shown but the standard errors cannot be calculated.


SJL
SPLEEN
MLN
BALB/c
CBA
TOTAL NO. CELLS (x 10⁻⁷)
0
2
4
6
8
10
0
4
6
13
35
WEEK POST INFECTION

In order to assess which cell populations were responsible for the enlargement of the organs the numbers of B cells were enumerated by using a fluorescein isothiocyanate (FITC) conjugated anti-mouse polyvalent immunoglobulin antibody. The numbers of T cells were counted by using a rat anti-mouse Thy-1 followed by a FITC-conjugated anti-rat IgG. Fig. 5.10 indicates that there was no appreciable change in the numbers of T or B cells in the spleens of any of the strains but the numbers of null cells (non T/B cells) in this organ increased quite dramatically.

Figure 5.10 Cell Types Present in the Spleen

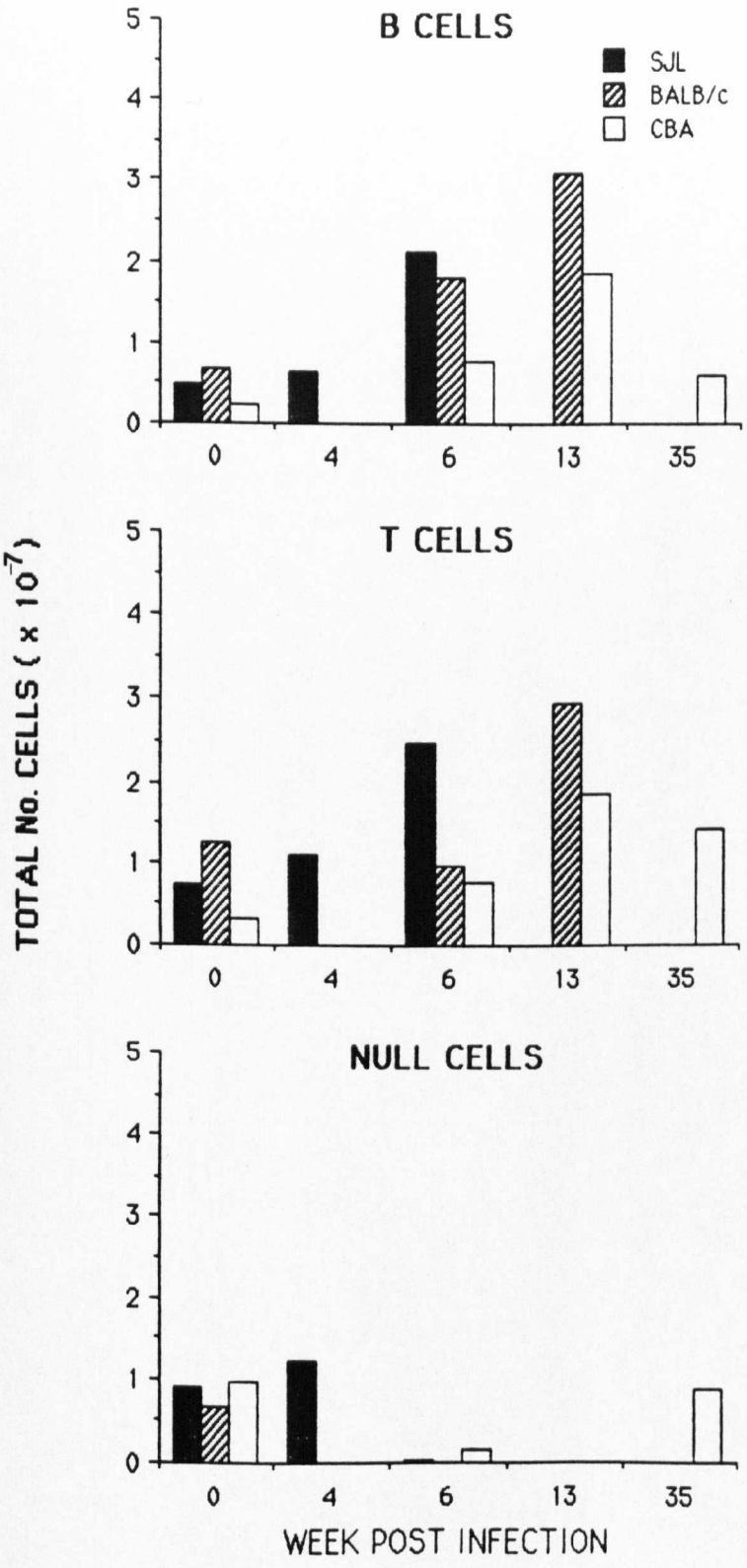
The total number of viable T, B and null cells present in the spleens of infected SJL, BALB/c and CBA mice was assessed by using FITC-conjugated antibodies directed against IgG,A and M (for B cells) and Thy.1 (for T cells). Those cells not labelled by either antibody were classed as null cells. As pooled cells were used the standard errors could not be calculated.


B CELLS
SJL
BALB/c
CBA
T CELLS
NULL CELLS
TOTAL No. CELLS (x 10⁻⁷)
WEEK POST INFECTION
0
4
6
13
35

The same data for the MLN is illustrated in Fig. 5.11. Both the numbers of T and B cells rose in this organ in response to the infection, with SJL responding better than either BALB/c or CBA, and in contrast to the events occurring in the spleen the numbers of null cells actually declined to zero in all of the strains.

Figure 5.11 Cell Types Present in the Mesenteric Lymph Node

The total number of viable T, B and null cells present in the MLN of infected SJL, BALB/c and CBA mice was assessed by using FITC-conjugated antibodies directed against IgG,A and M (for B cells) and Thy.1 (for T cells). Those cells not labelled by either antibody were classed as null cells. As pooled cells were used the standard errors could not be calculated.


B CELLS
SJL
BALB/c
CBA
T CELLS
NULL CELLS
TOTAL No. CELLS (x 10⁻⁷)
0
1
2
3
4
5
0
4
6
13
35
WEEK POST INFECTION

5.4.7 Blastogenic Response Of the Secondary Lymphoid Organs

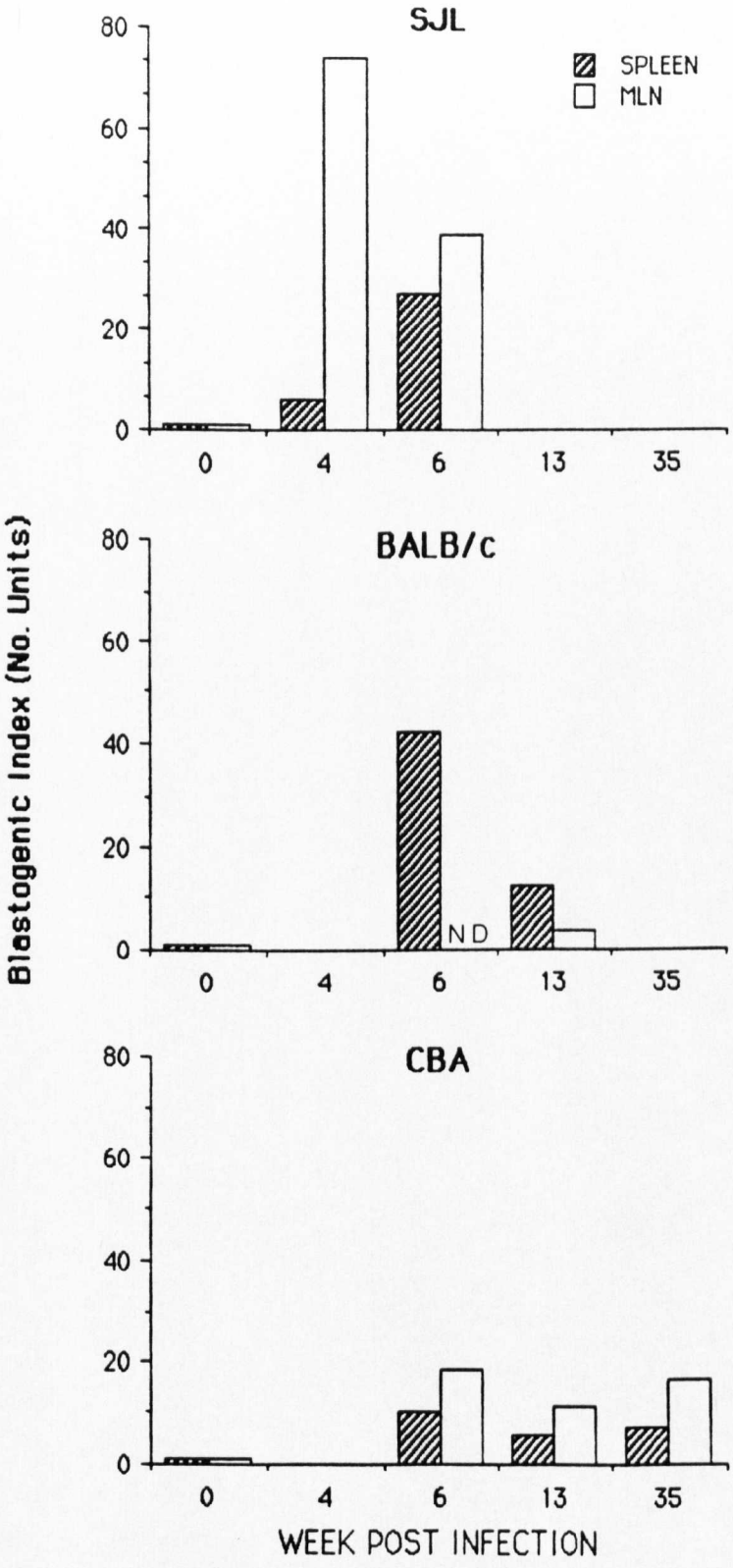
The blastogenic ability of the cells of these two organs was examined in order to determine if the expulsion of the parasite could be related to the responsiveness of the cells. In Fig. 5.12 the ability of the spleen and the MLN cells to proliferate is shown. The response of the spleen cells from SJL increased, CBA did not alter appreciably, whereas BALB/c rose and then fell. The blastogenic ability of the cells from the MLN did not alter markedly in BALB/c or CBA whereas those from SJL responded quite dramatically and to a much greater extent than those from the spleen.

Figure 5.12 Blastogenesis of the Cells of the Secondary Lymphoid Organs

The blastogenesis of the cells of the spleen and the MLN of infected SJL, BALB/c and CBA was assessed. Cells were taken at autopsy along with those from naive animals and cultured in medium containing 10% FCS. After labelling with tritiated thymidine, the blastogenic index was calculated thus;

$$\text{Index} = \frac{\text{Thymidine incorporation of infected cells}}{\text{Thymidine incorporation of naive cells}}$$

N.D = not done


SJL
SPLEEN
MLN
BALB/c
ND
CBA
Blastogenic Index (No. Units)
0
20
40
60
80
0
4
6
13
35
WEEK POST INFECTION

5.4.8 Response to Mitogen

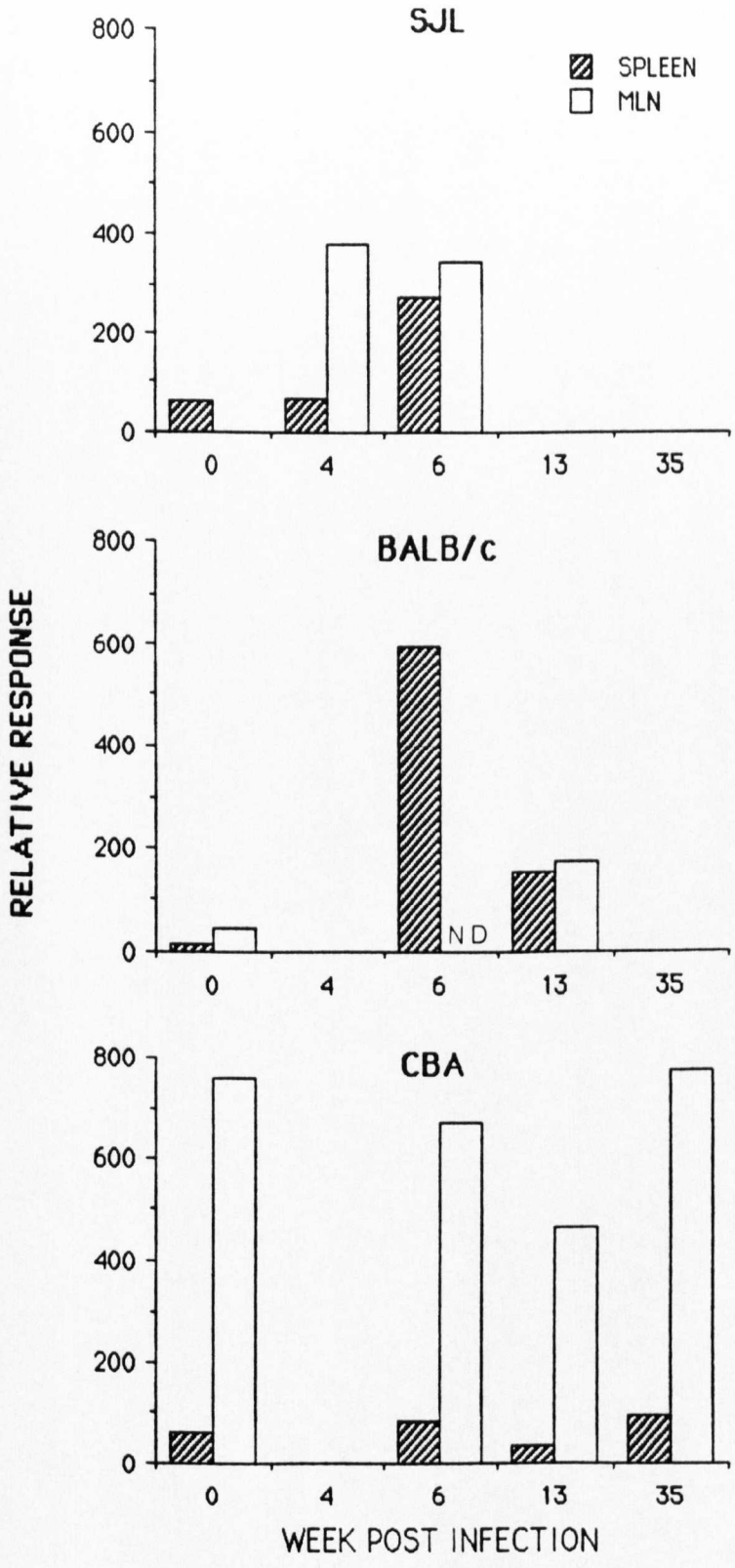
The response of cells from the spleen and MLN to two mitogens, Con A, a T cell phytomitogen, and LPS, a B cell mitogen, was examined. In Fig. 5.13, the relative response to Con A of the spleen cells did not alter in CBA, rose at week 6 and then fell in BALB/c and rose in SJL until the parasite was expelled. The response of the cells in the MLN rose slightly in BALB/c, but did not alter appreciably in CBA or SJL. However, the response of CBA was very high at all times, even at week 0.

Figure 5.13 Response of Cells from the Secondary Lymphoid Organs to the Mitogen, Con A

The response of cells from the spleens and the MLN of infected mice to the T cell mitogen Con A was assessed following culture of 5×10^6 cells with 1ug of Con A for 24 hours prior to the addition of thymidine at $37^{o}C$. The response of the infected cells relative to that of naive cells to the mitogen was calculated.

$$\text{Relative Response} = \frac{\text{cpm of mitogen stimulated cells}}{\text{cpm of unstimulated cells}}$$

N.D = not done


SJL
SPLEEN
MLN
800
600
400
200
0
0
4
6
13
35
BALB/c
ND
CBA
RELATIVE RESPONSE
WEEK POST INFECTION

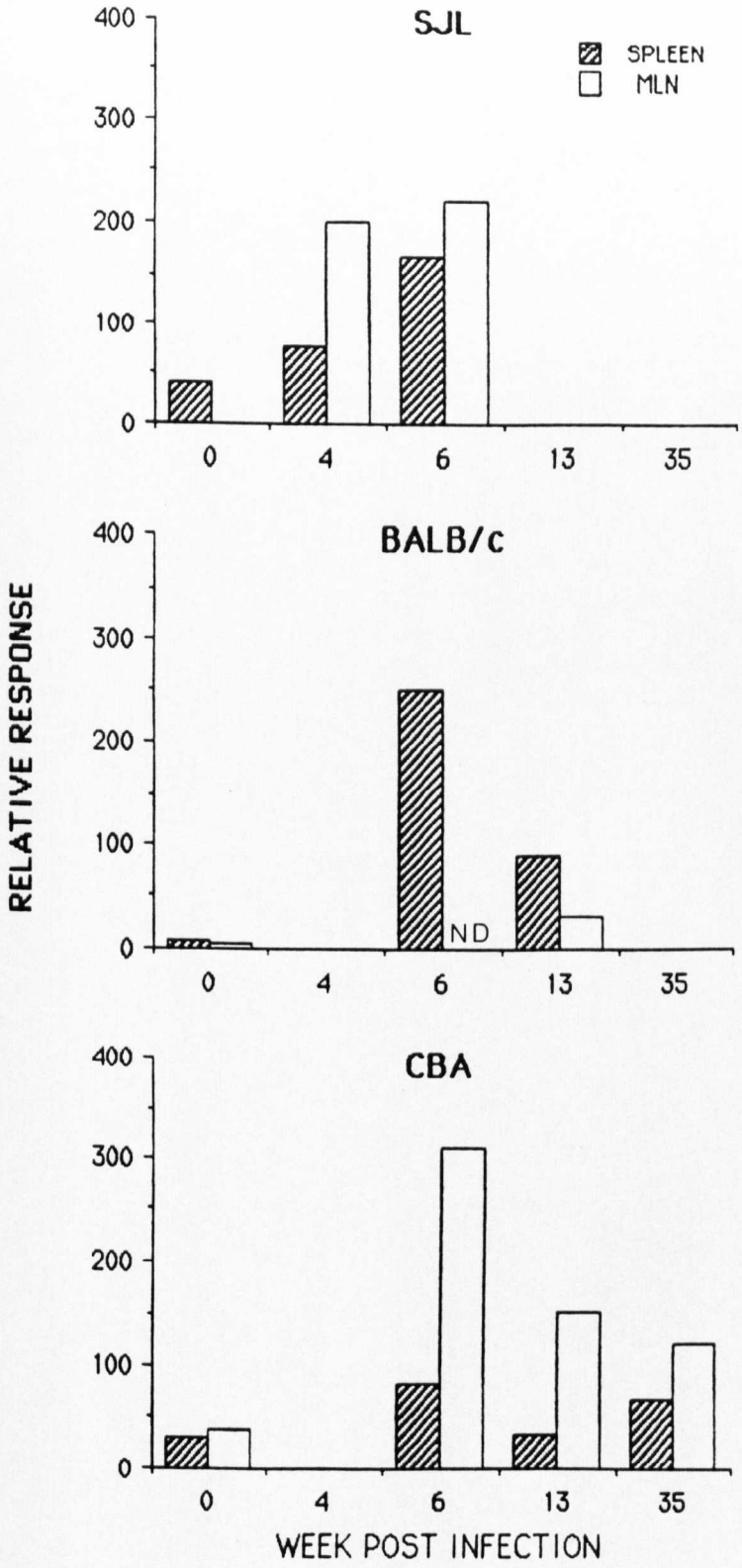
The response of the cells to LPS is shown in Fig. 5.14. The pattern of the response of the spleen cells is very similar to that seen in response to Con A. The response of the MLN cells to LPS is very similar in SJL and BALB/c to that to Con A, yet the response of the cells from CBA is quite different. The response peaked at week 6 and was lower overall than the response of SJL, with the sustained high levels of response noted with Con A not observed.

Figure 5.14 Response of Cells from the Secondary Lymphoid Organs to the Mitogen, LPS

The response of cells from the spleens and the MLN of infected mice to the B cell mitogen LPS was assessed following culture of 5×10^6 cells with lug of LPS for 24 hours prior to the addition of thymidine at $37^{o}C$. The response of the infected cells relative to that of naive cells to the mitogen was calculated.

$$\text{Relative Response} = \frac{\text{cpm of mitogen stimulated cells}}{\text{cpm of unstimulated cells}}$$

N.D = not done


SJL
SPLEEN
MLN
400
300
200
100
0
0
4
6
13
35
BALB/c
ND
CBA
RELATIVE RESPONSE
WEEK POST INFECTION

5.4.9 Mucosal Mast Cell Response

The importance of the gut mucosal response was evaluated by determining the numbers of mucosal mast cells.The mast cells were enumerated by staining fixed gut sections with an Alcian/Astra Blue stain. From Fig. 5.15 it can be noted that the numbers of mast cells per villus crypt unit (VCU) increased approximately seven fold in SJL and two fold in BALB/c but did not appear to increase in CBA.

Figure 5.15 Number of Mast Cells Present in the Gut

The numbers of mucosal mast cells present in the mucosa of infected SJL, BALB/c, and CBA mice was calculated by staining sections of gut obtained from each autopsy with Alcian/Astra Blue stain and counterstaining with Safranin O. The numbers of cells present in 25 villus crypt units (VCU) was counted per mouse and the mean and standard error for each group calculated.

SJL

Week 0 vs 4: $p < 0.005$ 0 vs 6: $p < 0.005$ 4 vs 6: $p < 0.005$

BALB/c

Week 0 vs 6: $p < 0.05$ 0 vs 13: NS 6 vs 13: NS

CBA

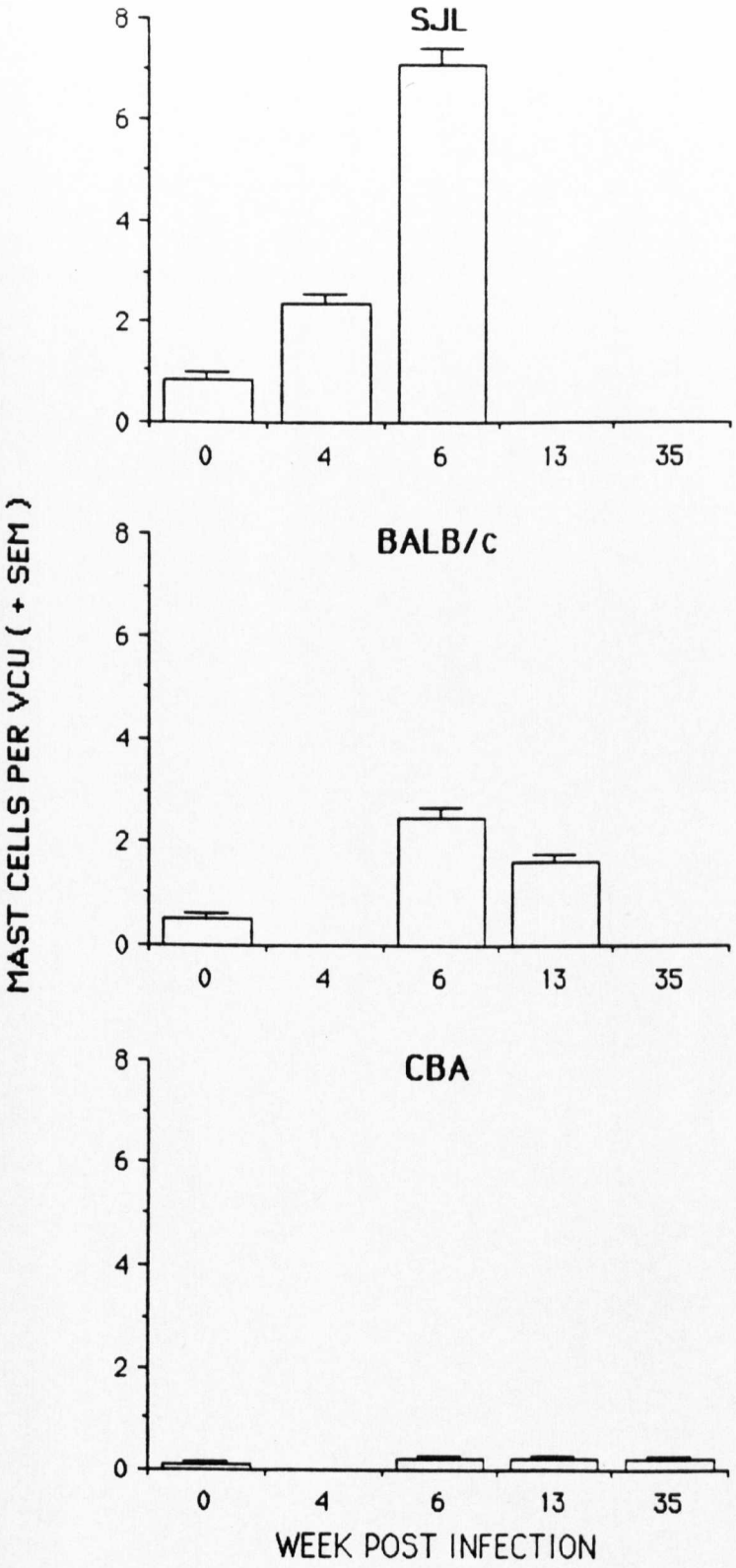
Week 0 vs 6: NS 0 vs 13: NS

Week 0 vs 35: NS 13 vs 35: NS

SJL6 vs BALB/c6: $p < 0.05$ SJL6 vs CBA6: $p < 0.005$

CBA6 vs BALB/c6: $p < 0.05$ CBA13 vs BALB/c13: $p < 0.05$

N.B. NS (not significant)


SJL
BALB/c
CBA
MAST CELLS PER VCU (+ SEM)
0
2
4
6
8
0
4
6
13
35
WEEK POST INFECTION

5.4.10 Antibody Response to Parasite Antigens

The enzyme linked immunosorbent assay (ELISA) is a major tool for the detection of antigens and antibodies in a wide variety of diseases. Components from the antigenic mixture attach to the surface of polystyrene plates by hydrophilic interactions and a monomolecular layer is formed with the amount of protein being bound proportional to the concentration in solution whilst at saturated concentrations a constant amount is bound. Partial purification of crude extracts through the use of excretory/secretory (ES) products can reduce the amounts of competing antigens and and thus permit coating of potentially more important antigens (Vogt, Phillips, Henderson, Whitfield and Spuerto, 1987). Non-specific binding of other protein components during subsequent steps is detrimental to the specificity and sensitivity of the assay. This non-specific binding may be minimised by saturating the remaining absorptive surface with blocking proteins that play no active role in the immunochemical reactions of the assays. The most effective agents are amphipathic proteins such as casein and lipoproteins. Instant milk (Marvel) provides a dispersible form of casein which binds quickly to the plastic plate and is readily available and cheap. However, there are disadvantages in using an ELISA as antibodies which may react in this assay may not necessarily react in a solution of antigen as binding to the plate may alter the conformation of the protein and thus expose epitopes unseen in solution (Vaidya, Dietzler and Ladenson, 1985).

Thus, in all ELISAs, the antigens used were parasite ES products either from adult or day 4 L4 parasites and skimmed milk (Marvel) was used as the blocking agent.

In order to determine the optimal antigen and antibody concentrations, dilutions of both antigens and antibody (immune and naive) were plotted in a chequerboard titration.

Fig. 5.16 shows the response of immune sera at a range of dilutions to a range of concentrations of adult and larval antigens. It can be seen that firstly, the adult ES was more antigenic than the larval ES and secondly, that a prozone occurred at all of the antigen concentrations tested. However, this only occurred with high antibody concentrations above a 1 in 32 dilution.

Figure 5.16 Chequerboard Titration of Sera and Antigens

In order to obtain the optimum conditions for an ELISA of the response of infected animals to parasite antigens a chequerboard titration of each was carried. The response of sera to a range of dilutions of adult and day 4 ES antigens was measured. The log 10 of antibody concentration against the various antigen concentrations was plotted.

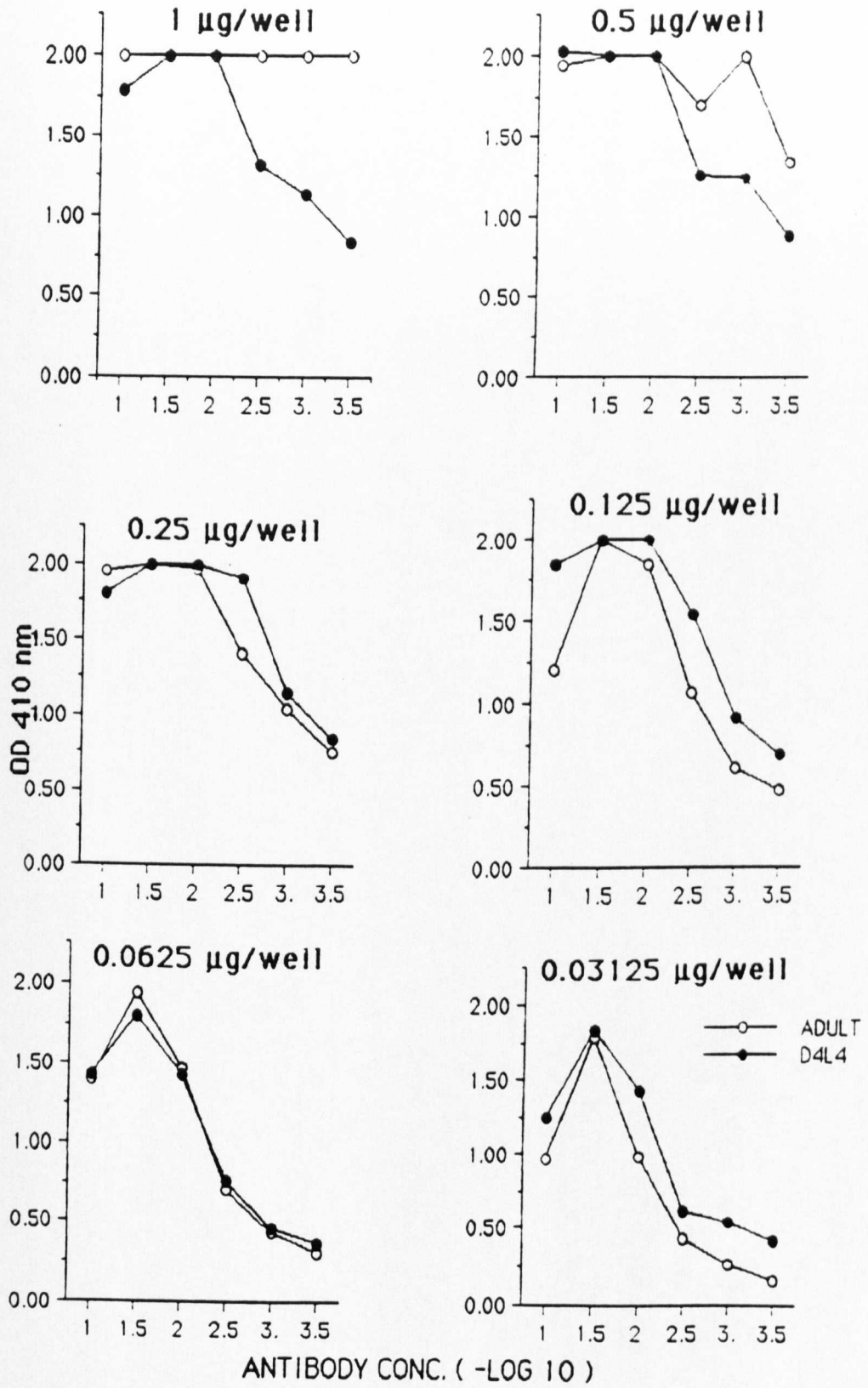

1 µg/well
0.5 µg/well
0.25 µg/well
0.125 µg/well
0.0625 µg/well
0.03125 µg/well
OD 410 nm
2.00
1.50
1.00
0.50
0.00
1
1.5
2
2.5
3.
3.5
ADULT
D4L4
ANTIBODY CONC. (-LOG 10)

Table 5.1 shows the immune:naive sera ratio of the chequerboard titration. These calculations indicate which antigen/antibody combination gives the best signal:noise ratio and thus indicates which are the optimal conditions to use. It was noted that for both the adult and larval preparations, the optimal antigen concentration was 0.25 ug per well whilst the optimal antibody dilution was 1 in 100. Thus these conditions were used in all subsequent experiments.

Table 5.1 Chequerboard Titration of Sera to Parasite Antigens

The ratio of naive:immune sera for each antibody and antigen concentration for both adult and day 4 L4 ES was calculated and plotted on a table. The optimal antibody dilution was 1:100, and the optimal antigen concentration was 0.25 ug/well for both antigens.

ADULT ES

ANTIGEN CONCENTRATION (µg / well)

ANTIBODY CONCENTRATION (LOG 10)	1	0.5	0.25	0.125	0.0625	0.03125
-1.0	1.62	1.22	**1.15**	0.92	0.98	0.81
-1.5	1.87	1.96	**2.00**	1.90	1.54	1.60
-2.0	**23.5**	**16.52**	**19.90**	**13.10**	**11.81**	**15.18**
-2.5	7.40	7.24	**7.35**	3.70	3.60	3.91
-3.0	6.37	6.09	**6.87**	5.69	5.00	3.95
-3.5	3.86	4.12	**4.22**	3.59	3.44	3.10

DAY 4 L4 ES

ANTIGEN CONCENTRATION (µg / well)

ANTIBODY CONCENTRATION (LOG 10)	1	0.5	0.25	0.125	0.0625	0.03125
-1.0	0.95	1.07	**0.89**	0.99	0.87	0.65
-1.5	1.58	1.53	**1.50**	1.56	1.24	1.01
-2.0	**5.80**	**7.19**	**7.50**	**6.74**	**5.66**	**3.61**
-2.5	4.30	3.50	**5.23**	5.30	3.16	2.67
-3.0	2.55	2.57	**3.04**	2.64	2.34	2.01
-3.5	2.47	2.78	**3.29**	2.81	2.15	1.91

Sera from various time points of the infection were tested against both adult and and day 4 larval ES products. It was decided to use day 4 L4 ES rather than the more widely used day 6 L4 ES as day 6 worms would contain both adult and larval parasites whereas day 4 worms would only contain third and fourth stage larval parasites and so would be more representative of a response against larval parasites. As can be seen from Fig. 5.17 the response to adult ES was strongest and most rapid in SJL followed by BALB/c and then CBA and appeared to follow the time course of the infection. The response to larval ES was negligible but followed the trends of the response to adult ES.

Figure 5.17 Antibody Response to Parasite Antigens

The quantitative antibody response of the sera from infected SJL, BALB/c and CBA mice to both adult and day 4 L4 ES was measured using an ELISA with the antibody concentration 1:100 and the antigen concentration 0.25 ug/well. The reaction was developed using alkaline phosphatase anti-mouse IgGAM. The mean and the standard error for each time point was calculated.

All Day 4 L4 ES: NS

Adult ES

SJL

Week 0 vs 4: $p < 0.05$ 0 vs 6: $p < 0.005$ 4 vs 6: $p < 0.05$

BALB/c

Week 0 vs 6: $p < 0.05$ 0 vs 13: $p < 0.05$ 6 vs 13: NS

CBA

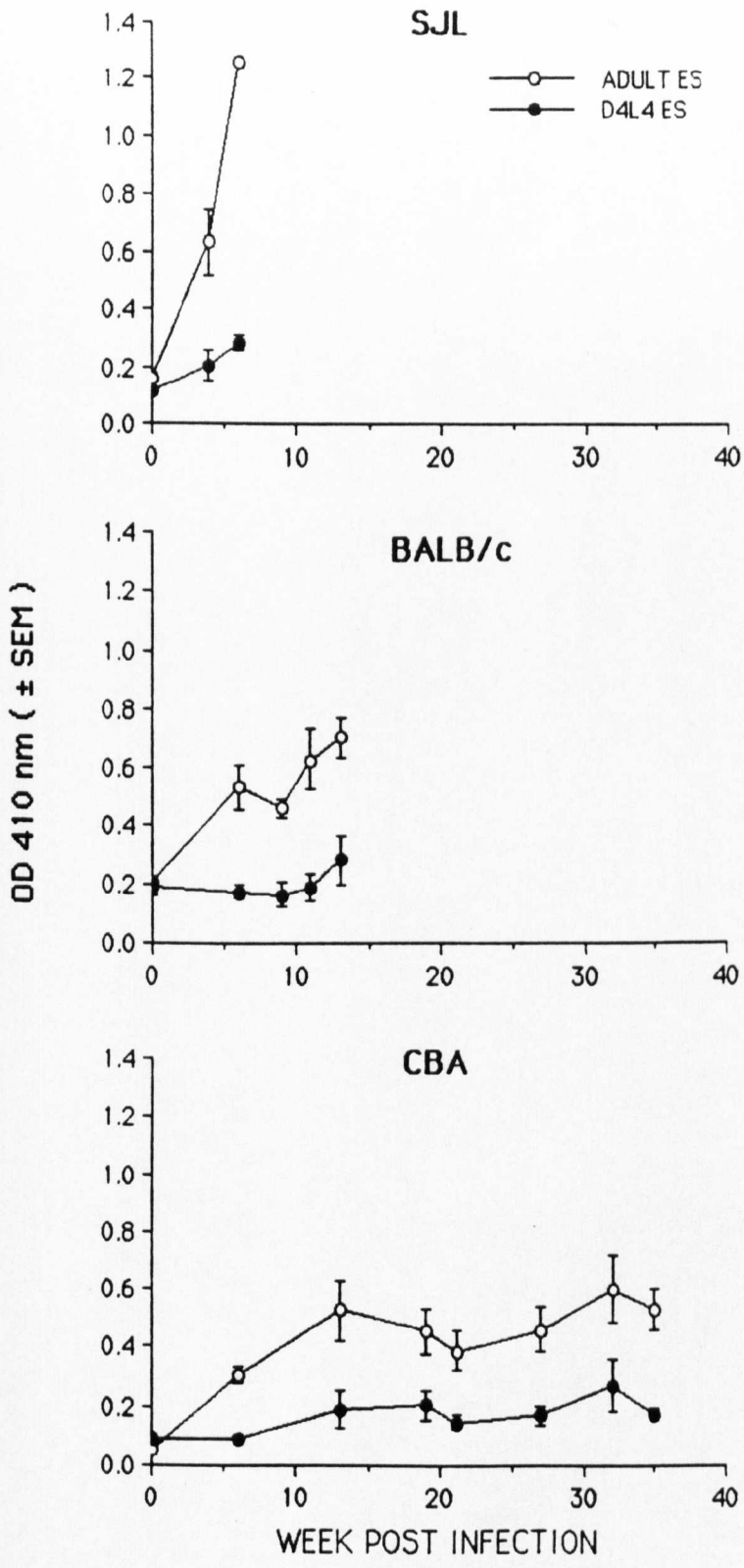
Week 0 vs 6: $p < 0.05$ 0 vs 13: $p < 0.05$

Week 0 vs 35: $p < 0.05$ 13 vs 35: NS

SJL6 vs BALB/c6: $p < 0.05$ SJL6 vs CBA6: $p < 0.05$

CBA6 vs BALB/c6: NS CBA13 vs BALB/c13: NS

N.B. NS (not significant)

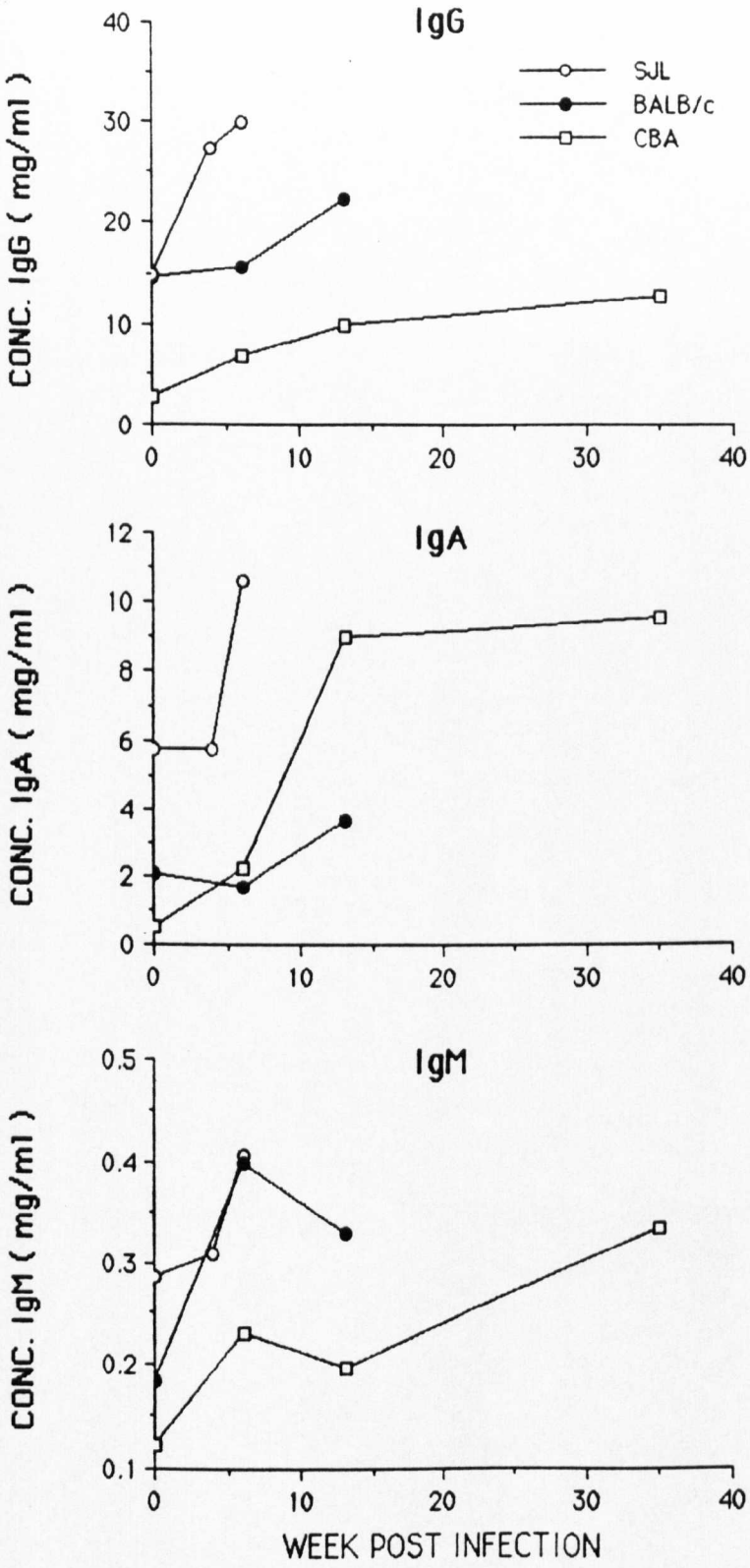

SJL
ADULT ES
D4L4 ES
BALB/c
CBA
OD 410 nm (± SEM)
WEEK POST INFECTION
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
10
20
30
40

5.4.11 Concentration of Antibody Classes

The antibody class predominating in the infection can be assessed by performing a Mancini test. The total antibody concentrations of IgG, IgM and IgA are illustrated in Fig. 5.18. It can be seen that the concentrations of all the antibody classes increased as the infection progressed. The levels in SJL were higher than in BALB/c which in turn, were higher than CBA and it appeared that IgG was the dominant class followed by IgA and then IgM.

Figure 5.18 Antibody Concentration

The concentration of the antibody classes, IgG, IgA and IgM was measured in infected SJL, BALB/c and CBA using single radial immunodiffusion kits (The Binding Site, Ltd.) for a number of time points during the infection. The concentrations of each are expressed as mg immunoglobulin per ml of serum.


IgG
SJL
BALB/c
CBA
CONC. IgG (mg/ml)
40
30
20
10
0
0
10
20
30
40
IgA
CONC. IgA (mg/ml)
12
10
8
6
4
2
0
0
10
20
30
40
IgM
CONC. IgM (mg/ml)
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
WEEK POST INFECTION

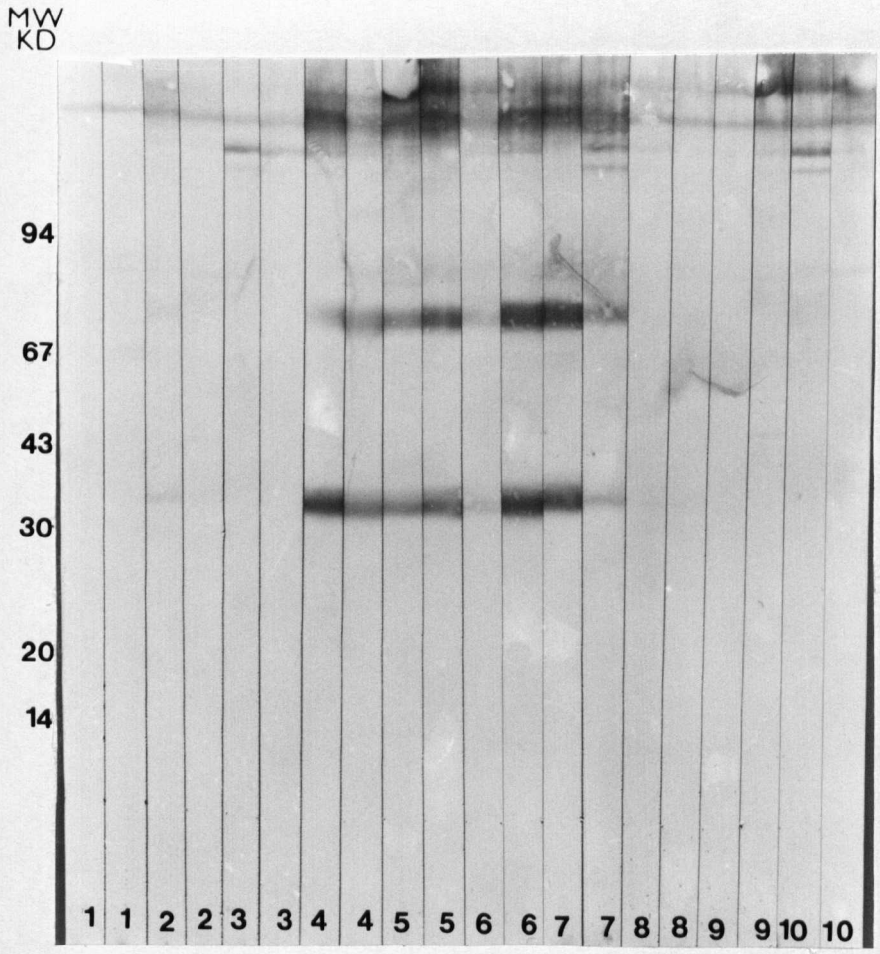
5.4.12 Qualitative Antibody Response to Parasite Antigens

As well as a quantitative analysis of the humoral response, a qualitative assay was performed by immunoblotting the various sera against adult ES and homogenate and day 4 L4 ES. It can be seen in Fig. 5.19 that SJL and BALB/c recognised two proteins of approximate molecular weight 67 and 30.5 kDa in parasite homogenate and this appeared to be related to the time of exposure.

Figure 5.19 Immunoblot of Adult Homogenate Probed with Infected Mouse Serum

1mg of day 10 adult homogenate was run under reducing conditions on a 5-20% SDS-PAGE gel. Following electrophoretic transfer to a nitrocellulose sheet, the resulting immunoblot was cut into 5mm strips and probed with the anti-sera at a dilution of 1:250 and developed with HRP-sheep anti-mouse IgGAM. Values for molecular weight standards are shown on the left hand side.

Lane	1	Naive SJL	
"	2	Naive BALB/c	
"	3	Naive CBA	
"	4	SJL	week 4 p.i.
"	5	SJL	week 6 p.i.
"	6	BALB/c	week 6 p.i.
"	7	BALB/c	week 13 p.i.
"	8	CBA	week 6 p.i.
"	9	CBA	week 13 p.i.
"	10	CBA	week 35 p.i.

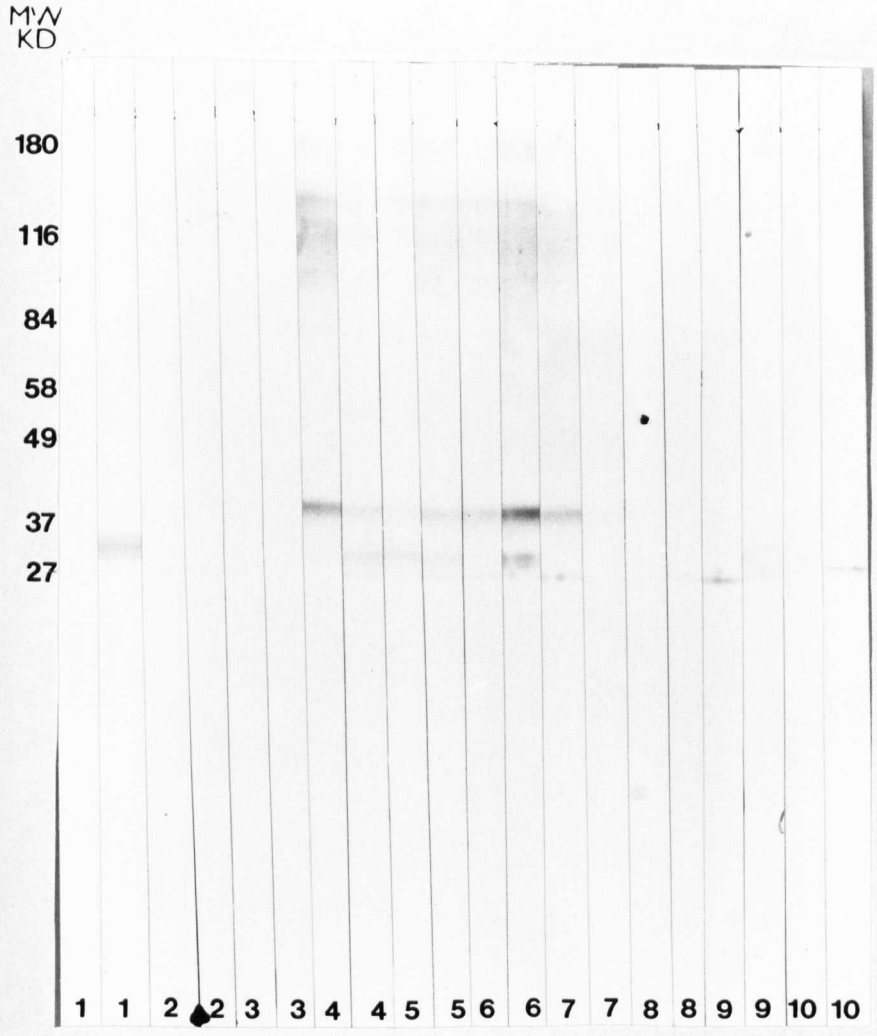

MW
KD
94
67
43
30
20
14
1 1 2 2 3 3 4 4 5 5 6 6 7 7 8 8 9 9 10 10

When the same sera were used to probe a blot of adult ES (Fig. 5.20) it can be seen that molecules were recognised of weight 137, 37 and 30.5 kDa and again this recognition appeared to be related to the ability of the strain to expel the parasite. These sera were used to probe a blot of day 4 L4 ES but no molecules were recognised by any of the strains.

Figure 5.20 Immunoblot of Adult ES Products Probed with Infected Mouse Serum

1mg of day 10 adult ES was run under reducing conditions on a 5-20% SDS-PAGE gel. Following electrophoretic transfer to a nitrocellulose sheet, the resulting immunoblot was cut into 5mm strips and probed with the anti-sera at a dilution of 1:250 and developed with HRP-sheep anti-mouse IgGAM. Values for molecular weight standards are shown on the left hand side.

Lane 1	Naive SJL		
" 2	Naive BALB/c		
" 3	Naive CBA		
" 4	SJL	week 4	p.i.
" 5	SJL	week 6	p.i.
" 6	BALB/c	week 6	p.i.
" 7	BALB/c	week 13	p.i.
" 8	CBA	week 6	p.i.
" 9	CBA	week 13	p.i.
" 10	CBA	week 35	p.i.


MW
KD
180
116
84
58
49
37
27
1 1 2 2 3 3 4 4 5 5 6 6 7 7 8 8 9 9 10 10

5.5 DISCUSSION

5.5.1 Faecal Egg Counts

The egg count data from this experiment revealed a number of points. The initial egg output of SJL was about one tenth that of BALB/c or CBA and the decline was much more rapid in SJL than BALB/c or CBA. This could be explained in a number of ways, firstly, less worms may have established in SJL and so overall the egg counts would be lower, secondly, immune responses could have acted from an early stage and caused a reduction in the fecundity of the worm, and lastly, the parasite may have been expelled earlier after infection.

5.5.2 Worm Burdens

When the worm recoveries at various time points were examined the establishment of the parasite in all strains was equal, the numbers of worms did not appear to decrease gradually but were expelled in a very short time period. SJL did not lose their parasites until about week 6 but their egg counts had declined long before this time. Therefore, it seems likely that an immunological response against the parasite caused a reduction in its fecundity.

Worm fecundity has been reported to be a fairly accurate estimation of worm vitality. Enriquez, Scarpino, Cypess and Wassom (1988) found that *in vivo* and *in vitro* egg production were correlated to each other and that faecal egg production reflected the numbers of adult worms present in the host. They proposed that reduced parasite fecundity was a manifestation of general anti-parasite immune responses rather than specific responses against worm reproduction. However, it would appear that this may not be the case in this instance as egg production declined before the parasites were completely expelled. Thus, there may be specific responses causing a reduction in parasite fecundity as well as general anti-parasite responses causing a reduction in worm vitality.

5.5.3 Worm Length

When the size of the parasites remaining at each autopsy was assessed, instead of the worms becoming smaller as expected, the average size increased. A possible explanation could be that those worms which remained in the face of an immune response were more robust, 'fitter' worms and thus would be more likely to survive, the damaged, stunted parasites having been expelled. However, those worms surviving in SJL were smaller than those in BALB/c or CBA suggesting that although they have managed to survive they may have been damaged and stunted by the host immune response. Those parasites remaining in CBA at week 35 were smaller than those at week 6 or 13 suggesting that either an immune response against the parasite had stunted its size or that the worm may be smaller due to natural senescence. This second hypothesis seems most likely as it has been demonstrated that this time period probably represents the natural life span of the parasite in the absence of an immune response (Behnke, Williams, Hannah and Pritchard, 1987).

5.5.4 White Blood Cell Counts

Examination of the peripheral cellular response revealed that the rise in cellularity appeared to be temporally related to the decline in faecal egg counts. This suggested that the decline in egg counts could be the result of an immune response against the parasite causing a reduction in its fecundity. The WBC response of SJL appeared more quickly and to a greater extent CBA. The rise in WBC number in CBA appears to be effective in reducing worm fecundity but does not appear to be sufficient to cause expulsion of the parasite. This could be due to a lack of effector cells and/or antibody necessary for adherence of the effector cells. A number of authors have indicated that there is an association between peripheral blood changes, namely, a general leukocytosis, and an immune response to *H. polygyrus* (Baker, 1962; Cypess, 1974; Prowse, Ey and

Jenkin, 1978; Behnke and Ali, 1985).

5.5.5 Differential Cell Counts

Differential cell counts carried out at these time points allowed a more detailed dissection of the response. The percent and total numbers of eosinophils did not appear to increase significantly in any of the strains in response to the infection. However, Behnke, Ali and Manger (1985) showed that there was an eosinophilia in CFLP mice infected with 400 *H. polygyrus* larvae, although this did not lead to expulsion of the parasite. The lack of eosinophilia noted in this infection could either have been due to the lower infection intensity or the strain of mouse used. It has been proposed for a number of years that the main cell population responsible for causing expulsion of, and damage to helminth parasites is eosinophils. Indeed, SJL are quite capable of producing an intense eosinophilia to parasitic infections such as *Toxocara canis* (Sugane and Oshima, 1985), but it is apparent that in an *H. polygyrus* infection this cell type does not mediate expulsion of the parasite. Although the precise function of the eosinophil in a parasitic infection has not yet been fully determined, two roles have been proposed. Firstly, they may be involved in the modulation or dampening down of the immediate hypersensitivity reactions attributable to IgE-dependent mast cell degranulation. Mast cell mediators are chemotactic for eosinophils, causing localisation of the cells and a dampening down of the mast cell reaction which would have been deleterious to the host. The second role for the eosinophil is the direct damage of the parasitic helminth. Antigens released from the parasite elicit a local IgE-dependent degranulation of mast cells which leads to the accumulation of antibody and complement around the parasite. This facilitates the adherence of eosinophils to the parasite. The cells then degranulate on the parasite surface and release their toxic contents disrupting the surface of the parasite. Examples of eosinophil mediated damage *in vitro* include damage of *Schistosoma mansoni*

schistosomula (Butterworth 1975; Butterworth et al 1977; McLaren, MacKenzie and Ramalho-Pinto, 1977; McLaren, Ramalho-Pinto and Smithers, 1978), *S. mansoni* eggs (James and Colley, 1978), microfilariae of *Litomosomoides carnii* and *Onchocerca volvolus* (Mehta et al, 1981; Greene, Taylor and Aikawa, 1981), larvae of *Trichinella spiralis* (Kazura, 1981; Grover, Butterworth, Sturrock and Bass, 1983) and microfilarie and larvae of *Dipetalonema viteae* (Haque et al 1981; 1982).

The development of eosinophilia appears to be controlled at the genetic level of the bone marrow derived cells, namely T cells (Vadas, 1982). However, there are instances where an eosinophilia occurs without the requirement for T cells. For example, an eosinophilia develops normally in athymic rats infected with *Ascaris suum* (Pritchard and Eady, 1981) or *Fasciola hepatica* (Day and Hughes, 1982). It has been shown that in rats infected with *Nippostrongylus brasiliensis* a sub-population of T cells, through the release of soluble mediators induces the expression and production by B cells of IgE specific for irrelevant antigens (Ishizaka and Ishizaka, 1978). This is thought to explain the potentiation of an IgE response without the presence of an eosinophilia. There appears to be no direct relationship between the development of an eosinophilia and the capacity to mount an IgE response in different strains of mice. An explanation for the presence of eosinophilia without damage to the parasite could be the result of some aberrance in the cell population, the cells could require activating in some way, or there could be a number of subpopulations, some of which are more potent in causing damage to the parasite, or there may be cells of different maturities or the eosinophilia could only consist of immature cells and thus, the parasite would be unharmed by the presence of these cells. In a primary infection of *H. polygyrus* it is thought that eosinophil killing is IgG_1 dependent (Prowse, Ey and Jenkin, 1978) and that by the time the relevant antibody response has been generated the parasite induced eosinophilia has died down and thus is ineffective in damaging the parasite. Eosinophils have been implicated as being involved in killing

trapped larvae in immune mice when an antibody response is present (Prowse, Ey and Jenkin, 1978; Hurley and Vadas, 1983).

When the percentage and total numbers of monocytes were examined there was little change although a slight drop may have been apparent. The most interesting change was the rise in the numbers of neutrophils, especially in BALB/c and CBA. This has been widely reported as a characteristic of this infection (Baker, 1962; Cypess, 1974; Prowse, Ey and Jenkin, 1978; Pentilla, Ey and Jenkin, 1983; Ali and Behnke, 1985). Pentilla, Ey and Jenkin (1983) studied the interaction between neutrophils and eosinophils and various stages of the parasite. It was demonstrated that mouse neutrophils and eosinophils could adhere to the different parasitic stages through the interaction of both Fc and C3 receptors with parasite bound antibody or complement and that the different stages showed varying degrees of cellular adherence, with attachment predominantly occurring to the early tissue stages of the worm. It was also demonstrated (Pentilla, Ey, and Jenkin, 1984a) that neutrophils and eosinophils from *H. polygyrus* infected mice exhibited larvicidal activity *in vitro*. Furthermore, the neutrophils required some kind of activation *in vivo* as those cells from naive mice had no effect on larvae even in the presence of complement and/or antibody, whilst those cells from infected mice had a potent larvicidal effect. These cells appeared in the blood 4 days after a primary infection and were still present at least 6 weeks later. The factors responsible for activating these cells were not elucidated nor was the need for an accessory cell population. It was also shown that these activated neutrophils were effective *in vivo*. When neutrophils were taken 4 days after a primary infection and injected into naive mice, protection was conferred as long as immune serum was donated within 24 hours of receipt of the cells, although serum alone did not confer any protection. Activated neutrophils required the presence of complement to protect the host but the activity of eosinophils was also dependent on the presence of IgG_1 antibodies for damage to occur *in vitro*.

Neutrophils have been implicated as being effective in

other parasitic infections. For instance, they can cause lesions in the tegument of *S. mansoni* (Incani and McLaren, 1983) and penetrate the cuticle and extend pseudopodia into the underlying tissues of second-stage larvae of *A. suum* (Thompson, et al, 1977). *In vitro* they have been effective against the microfilariae of *O. volvolus* (Greene at al, 1981) and *D. viteae*, in which they are the main effector cell type (Rudin et al, 1980). Neutrophil mediated adhesion in the presence of IgG has also been reported to damage *Wuchereria bancrofti* (Mehta et al, 1981). Even in a *T. spiralis* infection where eosinophils appear to be more active, neutrophils exhibited a strong larvicidal activity (Kazura and Aikawa, 1981).

Thus, it would seem that neutrophils are important effector cells in causing expulsion of *H. polygyrus*. However, SJL which are most effective at expelling the parasite, do not appear to have such a requirement for this cell population, as lymphocytes were the predominant population and the rise in the numbers of these cells appeared to be related to the expulsion of the parasite. An explanation for this phenomenon could be that the neutrophils from SJL were more active, or were activated earlier or that antibodies produced in conjunction with the cellular response were more effective or produced at an earlier stage in the infection than those in BALB/c or CBA, and so less of them would be required in order to expel the parasite. The increased lymphocyte population could be providing more accessory cells, colony stimulating factors and/or more antibody, thus enabling the neutrophils present to have a greater effect on the parasite. It is also possible that there are other effector cells, such as mast cells and eosinophils, in SJL, which are not present in BALB/c or CBA which act synergistically with the neutrophils and antibodies to cause destruction and expulsion of the parasite.

The macrophage is also believed to be an important component of immunity to this parasite and it has been shown to bind *in vivo* and *in vitro* to third stage larvae, impairing their infectivity (Chaicumpa, Jenkin and Fischer, 1977; Chaicumpa and Jenkin, 1978).

5.5.6 Cellular Response of the Secondary Lymphoid Organs

Examination of the secondary lymphoid organs, namely the spleen and the mesenteric lymph nodes, revealed that the increase in the size of the organs was the result of an increased cellularity, as reported by Ali and Behnke (1985) rather than oedema of the organ as suggested by Losson, Lloyd and Soulsby (1985). The total numbers of cells appeared to increase in both organs, in all strains, in response to infection, although the numbers in the spleen has fallen slightly in both SJL and CBA before the parasite was expelled. A splenomegaly in response to infection with this parasite has been reported in many studies (Baker, 1962; Price and Turner, 1983a; Ali and Behnke, 1985; Losson, Lloyd and Soulsby, 1985). Ali and Behnke (1985) established that the weight of the MLN increased rapidly in NIH mice, an intermediate responder, following infection with *H. polygyrus*, the spleen also enlarged but had returned to normal size once the MLN had achieved maximum size. In the low responder C57BL/10, enlargement of both organs was slower and the spleen did not regress in size. It was proposed that the reason for the regression of the spleen was because it was secondary to the MLN in importance and was only involved when the MLN were 'overloaded' with parasite products and required assistance from the spleen in dealing with this material. In the study by Losson, Lloyd and Soulsby (1985) it was demonstrated that the increase in the size of the spleen was proportional to both the cellularity of the organ and the infection intensity. However, in the MLN, the weight of the organ was inversely related to the infection intensity and did not correlate with the cellularity suggesting that the increase in the size was the result of oedema and that the response of this organ was secondary to that of the spleen. It was also noted as the spleen enlarged, the size of the MLN decreased, as reported by Hagan and Wakelin (1982) who noted a decrease in the size of the MLN early in the infection. However, when the spleen regressed the MLN enlarged so supporting the theory proposed by Ali and Behnke (1985).

Analysis of the cell population revealed more about the events involved in the expulsion of the parasite. The numbers of T cells in the spleen did not alter markedly in any of the strains studied and the number of B cells only rose fractionally in BALB/c and CBA. However, when the number of null cells was examined it was noted that there was a substantial rise in all strains in response to infection. The events occurring in the MLN were much simpler, with the rise in the total cellularity almost entirely accounted for by the rise in numbers of T and B cells with null cells declining and playing a negligible role in the enlargement of this organ.

The changes in the MLN can be explained quite simply, as the infection progressed there was a proliferation in the numbers of T and B cells which eventually led to the expulsion of the parasite. The B cells produced antibody against the parasite and the T cells could have mediated destruction indirectly through helper T cells and their cytokines recruiting B cells as well as effector cells such as neutrophils, eosinophils and mast cells. However, in the spleen the events were not so easy to interpret. There was no appreciable change in the levels of T and B cells but there was a large increase in the number of null cells, suggesting that this organ may have a slightly different role in the infection than the MLN. It was not clear as to the exact nature of these null cells, they are small mononuclear cells with no Thy.1 or antibody on the cell surface. Analysis of cell 'prints' from these organs confirmed that there were approximately 98% lymphocytes present with the remaining 2% consisting of granulocytes. Null, non T/B or third population cells are thought to be of a distinct lineage, carrying T cell markers at an early stage of differentiation and acquiring macrophage markers later. When mature, these cells assume the appearance of large granular lymphocytes. Most of them have receptors for the Fc portion of IgG and this population is particularly effective at killer (K) or natural killer (NK) activity. K cells are leucocytes which can recognise target cells coated with specific antibody: they bind via their Fc receptors and

subsequently kill the target cells. NK cells recognise determinants on some tumours and virally infected cells and can also kill them, although antibody is not required for recognition (Male, Champion and Cooke, 1987). As it appears that this cell population is maximal in this infection before but not during expulsion it could be that they are 'helping' the MLN cells to cope with parasite products as proposed by Ali and Behnke (1985), or that they are in the process of being activated before going on to the MLN and the gut. Again, it appears that the function of the spleen is secondary to that of the MLN and it serves to 'back up' the organ when it becomes overloaded with parasite material.

5.5.7 Blastogenic Response of the Secondary Lymphoid Organs

The blastogenic response of the cells of these organs tells us more about the responsiveness of the cells. It measures the spontaneous lymphocyte activity or the innate rate of proliferation of the cells. Increased responsiveness of cells from infected mice could be the result of a number of factors, including, a greater number of 'turned on' cells, cells that are more readily 'turned on',or the presence of parasite mitogen or immune complexes in the cell suspension which would stimulate the cells to proliferate. The cells from both the spleen and the MLN of CBA responded only slightly and this did not appear to be correlated with expulsion of the parasite. The MLN cells of BALB/c changed very little in their responsiveness and although the spleen cells became more responsive this did not appear to be related to loss of the worm. The cells from SJL showed high activity in both the spleen and the MLN and appeared to correlate with worm expulsion. Thus, it would seem that the expulsion of the parasite appears to be related to the excitability of the cells and that the more excitable they were, the more likely it was that the parasite would be rejected.

5.5.8 Response to Mitogen

A more detailed analysis on these cells involved their ability to proliferate in response to mitogen. Mitogens have the ability to induce blast cell transformation and mitosis in a manner similar to antigen. The mitogen binds to a specific cell-surface receptor, as does antigen, and the signal generated causes the nucleus to be de-repressed and the lymphocyte enters the cell cycle. Unlike antigens, however, mitogens stimulate a large proportion of lymphocytes but as for antigen stimulation of lymphocytes *in vitro*, there is an approximate correlation between the *in vitro* response to mitogens and the immune status of the individual.

Overall, the response to LPS, a B cell mitogen, was much lower than that to Con A, a T cell mitogen. This suggested that T cells were playing a more important role in the generation of immunity to the parasite than B cells. The response of both the spleen and MLN cells to LPS rose in SJL, suggesting that both the number and the reactivity of the cells had increased. The response of the spleen and MLN cells to LPS in BALB/c and CBA did not seem to relate to the numbers or reactivity of the cells implying that either the B cell response was not active or was being suppressed. The cells may also be already committed and so will not proliferate in response to the mitogen. Prior stimulation of cells by *H. polygyrus* antigens to produce antibody or stimulation or amplification of clones of cells to produce antibody to unrelated antigens has been postulated to account for the general lack of responsiveness to LPS of the already committed cells (Coutino and Moller, 1975; Corsini et al, 1977). The high responsiveness of MLN cells from CBA to Con A suggests that either the T cells are non-functional, T-suppressor cells or that they are defective and not capable of driving an effector or B cell response. It appeared that the MLN was a more responsive organ than the spleen, in this situation, and that the T cell population could be more important in expelling the parasite than the B cell population, as long as the cells

were functional.

Losson, Lloyd and Soulsby (1985) found that the responsiveness of the spleen and MLN cells to mitogens altered as a result of infection with *H. polygyrus*. There was a reduced ability of the MLN to respond to PHA and Con A (T cell phytomitogens). This could have been due to migration of cells to the spleen, as this became more responsive, although proliferation of cells was also thought to occur. Responsiveness to LPS, a B cell mitogen, was suppressed in the spleen, although there was B cell proliferation as assessed by histology and antibody production. Sheep and mice lymphocyte proliferation to all mitogens was suppressed by both a larval product of *H. polygyrus* and serum from infected mice suggesting that the parasite was able to suppress both the activity of T and B cells. Other helminths have also been shown to produce factors *in vitro* which inhibit mitogen induced lymphocyte transformation (Dessaint et al, 1977; Ouassi et al 1983; Price and Turner, 1986b; Garside et al,1989). In this study there was no reduction in the ability of cells to respond to mitogen at any stage of the infection studied. The differences may have been due to the strain of the host, the incubation period or the sampling times. By the time this first sample was taken the previous study had shown the responses to be normal and had not studied them further.

5.5.9 Mucosal Mast Cell Response

Parasitic gastrointestinal nematodes invariably cause a mucosal mastocytosis which appears to be correlated with their expulsion from the host. Mucosal mast cells (MMC) arise from bone marrow precursors after stimulation with a T cell derived factor thought to be IL-3, although other components may also be involved. For instance, expulsion of *N. brasiliensis* in rats (Nawa and Miller, 1979), *Trichostrongylus colubriformis* in guinea pigs (Rothwell and Dineen, 1972), *Strongyloides ratti* in rats (Olson and Schiller, 1978) and *T. spiralis* in mice (Alizadeh and Wakelin, 1982a) is correlated to a mucosal

mastocytosis. *S. ratti* in W/W^V mice, a mast cell deficient strain, is not expelled unless the animals are reconstituted with mast cells. However, there are some cases in which expulsion occurs in the absence of a mastocytosis e.g. W/W^V mice will expel *N. brasiliensis* although it is slightly delayed (Uber, Roth and Levy, 1980), mice treated with 5-hydroxy tryptamine (5-HT) (serotonin), which suppresses a response, still expel *T. spiralis* (Parmentiers, de Vries, Ruitenseg and van Loveren, 1987) and adoptively immunised irradiated rats still expel *N. brasiliensis* (Ogilvie, Love, Jarra and Brown, 1977). Conversely, the other situation also occurs, *N.brasiliensis* in neonatal or lactating rats is not expelled despite a mastocytosis (Jarrett, Urquhart and Douthwaite, 1969; Kelly and Ogilvie, 1972) and maximum accumulation of mast cells in normal rats and mice occurs after the parasite has been expelled.

The problem with the examination of histological sections is that not all of the mast cells are revealed. For instance, immature cells which probably predominate and are as effective as mature cells, and cells which have been activated and have lost their granules, do not stain, thus, staining gives no idea of the functional status of the cells. These numerous membrane bound cytoplasmic granules contain proteoglycans, histamine and proteases which are released when the cell is activated. Rat mast cell protease II (RMCP II) is one of these proteases. It is a neutral proteinase which is released from functional immature and mature mast cells and provides a more accurate assessment of MMC activity. By measuring RMCP II levels in the serum of *N. brasiliensis* infected rats (Woodbury et al, 1984) it was found that there was a rise in levels prior to any significant increase in mast cell counts. Conversely, when serum RMCP II levels fell, the MMC counts had risen substantially. Although there were a large number of stained granules in these cells it was suggested that they were functionally inactive.

Mast cells are thought to act in a number of ways. Release of soluble mediators may allow translocation, into the

gut lumen, of antibodies and complement by altering the mucosal permeability, they may also alter the epithelial integrity, secretory activity and/or act on smooth muscle cells of the intestine thus altering the intestine in a way which may cause the parasite to become dislodged. The contents of the mast cell granules may also affect the parasite directly by causing disruption of its cuticle (McKean and Pritchard, 1989). It has also been proposed that the role of the mast cell may also be regulatory as it may stimulate the production of mucus resulting in the mechanical expulsion of the worm, as well as delivering antibody and complement to the parasite. Other cells which are not detected, such as basophils, enterochromaffin cells and platelets may also play a role in the anaphylactic reaction and cause expulsion of the parasite in much the same way as mast cells.

The results of this study suggest that an *H. polygyrus* infection does produce a significant mastocytosis in SJL mice and a slight one in BALB/c but no response is elicited in CBA. This is in contradiction to the results of the study by Dehlawi and Wakelin (1988) where no mastocytosis was demonstrated in a primary infection in SJL, NIH or B10G mice, although a strong response was noted in a challenge infection in SJL. It is well known that *H. polygyrus* suppresses the mastocytosis produced by other parasitic infections such as *T. spiralis* and *Trichuris muris*. It was demonstrated (Dehlawi, Wakelin and Behnke, 1987) that a concurrent infection with *T. spiralis* resulted in the delay and/or depression of the mastocytosis associated with the expulsion of this parasite. They further went on to show that it was the adult stage of *H. polygyrus* which suppressed the mucosal mast cell response and concluded that some parasite factor prevented the generation of lymphocytes capable of stimulating the development of MMC from precursor cells (Dehlawi and Wakelin, 1988). An *in vitro* study (Reed, Dehlawi and Wakelin, 1988) showed that 18 day infected mouse spleen cells were not able to support cultured mast cells whereas those from day 6 infected mice were. It was proposed that the adult worm had the ability to alter production of lymphokines

in spleen cell suspensions which promote the development of MMC from their precursors.

Thus, although a small, but significant, number of mast cells were observed, a functional mast cell response could be occurring. As the staining technique does not reveal all of the functional cells the extent of the reaction is not known. A much more accurate assessment would be to analyse the RMCP II equivalent levels in the serum of infected mice and this is currently being investigated by other members of the laboratory.

5.5.10 Antibody Response to Parasite Antigens

The results of the ELISA again showed that SJL responded quicker and to a greater extent than the other two strains, although there was no response to larval antigens in this or the other strains. Therefore, it was noted that there was strong antibody response against adult parasite antigens suggesting that this may have been responsible for the rejection of the parasite. The fact that adult ES was recognised whilst larval products were not suggests that there are few cross-reacting larval and adult antigens, thus confirming the work seen in Chapters 3 and 4. It is also possible that in a primary infection adult antigens are more immunogenic than the larval proteins and so will be recognised more readily. It is not entirely surprising that expulsion of the parasite is not correlated with the recognition of larval proteins as the host will only have experienced these for a maximum of 7 days whereas adult proteins would have been present for at least 6 weeks, even in the high responder strain.

5.5.11 Concentration of Antibody Classes

When the class of antibody predominating in the serum of infected mice was analysed and quantified, it was seen that IgG was present in the greatest amounts, confirming the work of previous authors (Pritchard et al, 1986; Shimp, Crandall and Crandall, 1975; Knopf et al, 1979).

5.5.12 Qualitative Antibody Response to Parasite Antigens

When various antigen preparations from *H. polygyrus* were probed with sera on a Western blot, it was noted that a number of molecules from adult preparations were recognised although none were seen in larval preparations. Again this is not entirely surprising due to the reasons described above. It was also seen that different molecules were recognised in adult homogenate and ES. The 30.5 kDa molecule seen in both preparations may be the same molecule. It is known that surface molecules from nematode surfaces are actively shed into culture medium and this may represent one of these molecules. The 67 kDa molecule recognised in the homogenate could be the same as that seen on the surface of adult parasites (see Chapter 4), as this is present in large amounts on the parasite surface and is recognised strongly on immunoprecipitation (see Chapter 7).

The 30.5 kDa protein recognised in adult ES may be the AChE which was shown to be present in both adult and larval ES and which was recognised by a rabbit anti-*Necator americanus* AChE (see Chapter 3). Although this molecule is present in greatest amounts in the larval parasite, it may still be important to the adult worm in delaying its expulsion in conjunction with other immunomodulators. Hence recognition of this molecule by the host could have serious consequences for both the adult and larval parasite, resulting in not only paralysis of the worm but a neutralisation of its 'biological holdfast' and may be even ablating its potential immunosuppressive effect.

Recognition of the surface molecules would clearly have the capability of causing severe disruption to the parasite cuticle and thus result in expulsion of the worm. If a greater number of molecules are recognised this would clearly damage the parasite in a greater number of ways and thus be more likely to result in its rejection from the host.

It was very interesting to note that CBA recognised little or no molecules at any stage of the infection, even when parasite loss occurred. Again, this implies that expulsion of the parasite from this host strain is more likely to be due to parasite senescence rather than a response from the host.

The contribution of the humoral response to immunity in *H. polygyrus* has been extensively studied by a number of authors. Panter (1969) reported that mice immunised with two infections of *H. polygyrus* precipitated one line on immunoelectrophoresed adult homogenate whilst none was precipitated by mice subjected to a primary infection. When exsheathed L3 were placed in immune sera a precipitate developed around the excretory pore although the infectivity of the larvae was not affected. Bartlett and Ball (1974) proposed that immunity to the parasite consisted of both a humoral and cellular component. Serum donated from immune mice to naive recipients was capable of delaying the development of the larvae, possibly by preventing them from penetrating the mucosa, although no effect was seen on the adult parasite with its fecundity being unaffected.

Studies by Behnke and Parish (1979) showed that immune sera transferred to naive recipients severely impaired the survival of the parasite. Not only were larvae lost early on but their development was inhibited and the worms were stunted. However, expulsion of the parasite was not initiated which suggested that although the sera were capable of damaging the worms another factor was required for their expulsion. It was further demonstrated (Behnke and Parish, 1981) that MLN cells were instrumental in immunity, although both cells and serum alone were able to cause damage of the parasite, given together they acted synergistically. There was a greatly enhanced early loss phase which suggested co-operation between the two, and it

was put forward that the sera was capable of damaging the parasite but a constituent of the cell population was responsible for the loss and expulsion of the parasite. Williams and Behnke (1983) demonstrated that host protective antibodies first appeared in the serum of immunised mice 3 to 4 weeks after the beginning of immunisation and was maximal after 6 weeks. It was demonstrated that serum from C57BL/10 (a low responder) was as effective as that from CFLP, but that of NIH (a high responder) was not as efficient. It was thought that the reason for this could be that the NIH had a better cellular response than the other two strains and thus was better at killing the parasite, or that the quality of the serum was different in some way, making it more effective at eliciting a cellular response against the parasite.

Dobson (1982) indicated that passive transfer of immunity to mice with sera was proportional to the number of infections of the donor, the antibody titre and the quantity of the serum. The worms in the recipient mice were affected in a number of ways, their survival was reduced, the length of the worm and its fecundity were reduced. Male parasites were more severely affected than females. Immune sera from female mice was better than that from males and female recipients were better protected than their male counterparts, thus, suggesting a role for sex hormones in the control of immunity to the parasite.

Chaicumpa, Jenkin and Rowley (1976) showed that immunity could be passively transferred to a neonatal animal but not to an adult. The immunity was demonstrated to be transferred after birth and not *in utero* and so they concluded that it was due to IgA in the colostrum which would only be present in small amounts in the serum. Molinari, Ebersole and Cypess (1978) also showed that specific antibodies were present in the IgA fraction.

Behnke, Williams, Hannah and Pritchard (1987) showed that the levels of IgG_1 in a primary infection were highest in mice with the highest worm burden but had declined by the time the worms were expelled. In attempts to characterise this response further the class of antibody involved in immunity was

analysed. It was found that in a primary, chronic infection the levels of IgG_1 rose after 2 weeks and stabilised at double the normal levels, however, immunity could not be transferred using this serum (Crandall, Crandall, and Franco, 1974; Molinari, Ebersole and Cypess, 1978; Shimp, Crandall and Crandall, 1975; Prowse, Ey and Jenkin, 1978; Chapman, Knopf, Anders and Mitchell, 1979). It was further demonstrated that the hypergammaglobulinaemia was composed of a heterologous population of IgG_1 molecules. Williams and Behnke (1983) found no rise in the antibody response in a primary infection. However, in an immunising infection they found a major change in IgG_1 levels proportional to the rise in the host protective response.

A hypergammaglobulinaemia was also reported by Pritchard Williams, Behnke and Lee (1983). It was demonstrated that that there was a 10 fold increase in the IgG_1 levels of immunised mice but a decrease in the levels if IgG_{2a} and IgG_{2b}. Additionally, it was proven that 48% of the IgG fraction consisted of adult specific antibody whilst the remainder, but not all, had some response to fourth stage larvae. The IgG_{2a}, IgG_{2b} and IgG_3 fractions had no anti-parasite activity. The IgG_1 reacted with both homogenate and ES antigens and recognised molecules of 17, 18 and 20 kDa. *In vivo*, the IgG_1 fraction caused a stunting of the parasite. An explanation for the decreased $IgG_{2a,\ 2b\ \&\ 3}$ levels despite a dramatic rise in IgG_1 could be explained by an increased catabolic rate. Catabolic rates of all IgG classes are accelerated by increased serum levels of any IgG class (Fahey and Sell, 1965). Brown, Crandall and Crandall (1976) found a decrease in the IgG_1 half life in *H. polygyrus* infected mice consistent with the theory that the increased IgG_1 levels caused an elevation in the catabolic rate. They also suggested that the increased catabolism would cause the apparent suppression of the antibody response as effective antibodies would be quickly catabolised.

Elevated IgG_1 levels have been noted in other parasitic infections e.g. *Echinococcus multilocularus*, where 92.6% of the serum IgG_1 was parasite specific (Ali-Khan and Siboo, 1982),

Leishmania tropica (Liew, Hale and Howard, 1982) cestodes (Mitchell, Goding and Rickard, 1977; Mitchell, Marcholonis, Smith, Nicholas and Warner, 1977) and *S. mansoni* (Sher, McIntyre and Lichtenberg, 1977).

The reasons for the presence of this IgG_1 have not been fully elucidated, but a number of mechanisms for its generation have been proposed. Liew, Hale and Howard (1982) suggested that high IgG_1 levels were a consequence higher antigenic stimulation, as a result of increased parasite burden following suppression of the immune response by the parasite. Shimp, Crandall and Crandall (1975) also indicated that the high persistent levels of IgG were due to sustained antigenic competition. Another proposal was that they blocked potentially protective host responses by 'exhausting' the B cells by mass clonal expansion to irrelevant antigens (Mitchell Marchalonis, Smith, Nicholas and Warner, 1977; Mitchell et al 1982). Price and Turner (1983a) suggested that the spontaneous rise in SRBC titres in *H. polygyrus*, *T. muris* and *N. brasiliensis* infected mice was due to cross reacting antigens entering from the gut which were mitogenic to B cells. In another study (Price and Turner, 1983b) it was showed that an increased IgG and IgM response to PVP administered i.p. to infected mice. It was put forward that the enhanced T helper independent antibody response by systemic contact with the parasite material could be due to hepatic or alveolar macrophage activation and that suppressor T cells were activated by persistent circulating antigen.

Parasite proteins with direct suppressor activity are distinct from parasite mitogens that might indirectly contribute to immune suppression via polyclonal activation of the host immune system. This could explain the apparent paradox of polyclonal hypergammaglobulinaemia in infections where there are no specific antibodies to parasite antigens (Wadee, Vicky and Piessens, 1987).

There is some debate as to whether the IgG_1 hypergammaglobulinaemia is protective, in some cases it is, in others it appears not to be. The first point to note, is if

IgG_1 actually reaches the parasite in the gut lumen. Cypess, Ebersole and Molinari (1977) showed that the concentration of IgG in the gut lumen increased substantially 3-7 days p.i. and so it seems that it would be the case in this infection. It has also been implied (Behnke, Williams, Hannah and Pritchard, 1987) that in a primary infection the production of antibodies led to the parasites inhabiting a sub-optimal environment in the inflamed lumen and thus the parasite may sustain damage through an inability to respire and/or feed normally.

Attempts have been made to further characterise the response by the production of monoclonal antibodies. Two monoclonal antibodies have been produced (East, Washington, Brindley, Monroy and Scott-Young, 1988). Though they did not reduce the worm burden when transferred to naive recipients, one depressed egg counts and the length of the worms equivalent to the effects of immune sera. The other had no effect on egg counts but it did stunt the parasites. Unfortunately, the molecules being recognised were not characterised.

IgG_1, like IgE, is homocytotropic and will sensitise mast cells for degranulation in the presence of antigen and will mediate eosinophil adherence to the helminth. IgE is thought to be the most important antibody class in parasitic infections. Increased IgE levels have been associated with *A. lumbricoides* (Johanssen, Mellbin and Vahlquist, 1968) *Toxocara* spp. (Hogarth-Scott, Johanssen and Bennich, 1969) *T. spiralis* (Rosenburg, Pulmar and Whalen, 1971) *Capillaria phillipensis* (Rosenburg, Pulmar and Whalen, 1971), *S. japonicum, W. bancrofti* and hookworm (Ito, Sawada and Sato, 1972). The importance of this in protecting the host is debatable. It has been shown that IgE has a function to induce eosinophilia (Kay and Austen, 1971) and protective immunity (Butterworth, 1977; Capron, Capron and Dessaint, 1980). In the case of these two infections it is known that SJL are better responders than BALB/c even though they have a similar IgE response and eosinophilia. It was proposed that this was because of a differential sensitivity to vascular amines. Vasoactive amines liberated as a consequence of anaphylactic reactions involving

IgE could cause mediators, responsible for worm elimination, to pass more freely through the tissue and thus would be more reactive to the parasite. However, no differences in response were found when mice were injected with histamine.

An eosinophilia independent of IgE antibody has been reported. It has been demonstrated that IgE is not necessary for the development of eosinophilia or protective immunity in *N. brasiliensis* and *T. spiralis* infections (Watanabe, Katakura, Kobayashi, Okumuro and Ovary, 1988), and it is proposed that eosinophilopoietin, eosinophil stimulation promoter and IL-5 released from T cells may be responsible (Warren, Karp, Pelley and Mahmoud, 1976; Mahmoud, Store and Tracy, 1979).

In general it can be seen that in this *H. polygyrus* infection the greater and swifter the response was to the parasite, resulting in a quicker expulsion of the parasite, the better the strain was at reacting to the parasite . The response to the parasite was greater on both the humoral and cellular levels, the antibody response to the parasite was greater both quantitatively, as seen by ELISA and Mancini tests, and qualitatively, on the Western blots. The increased cellular response could be observed at both the peripheral level and closer to the site of infection, i.e. an increased cellularity of the secondary lymphoid organs and a mucosal mastocytosis in the gut.

The high responsiveness of SJL and low responsiveness of CBA in response to infection with *H. polygyrus* could be related to the phenotype of the major histocompatibility complex (MHC). Genes encoded within the I region of the mouse H-2 complex encode polypeptides which provide a context for the recognition of antigen by T cells. Most inbred strains of mice express two I region products I-A and I-E, on the surface of antigen-presenting cells. However, strains with the s, q or f haplotypes of H-2 fail to express the I-E product, whereas others do. In this case SJL (s haplotype) do not express the I-E whereas BALB/c (d haplotype) and CBA (k haplotype) do.

Wassom, Krco and David (1987) proposed a mechanism for the control of resistance to *H. polygyrus* in mice. In experiments using congenic mice of different H-2 haplotypes they found that resistance was very similar to that seen in infections with *T. spiralis*. In this, if susceptible B10.BR mice H-2^k, I-E^+) were crossed with resistant B10.Q or B10.M (H-$2^{q,f}$, I-E^-); the offspring were susceptible to *T. spiralis*. Therefore, mice which had the s, f or q haplotype were resistant to infection and those with the k haplotype were susceptible. Thus, it appears that expression of the I-E molecule determines the susceptibility of the host to the parasite. Recognition of relevant parasite antigens in association with I-A molecules on the surface of antigen-presenting cells stimulates the T-h cells to proliferate and provide help to other T cells and B cells and thus effect a functional anti-parasite response. Parasite antigens presented in the context of I-E molecules are thought to activate T-suppressor cells which down regulate the I-A restricted response. I-E restricted, antigen specific T cells would have to induce auto-reactive cells which would down regulate the I-A restricted anti-parasite response in I-E^+ mice. I-A restricted T cells failed to persist in *in vitro* cultures where I-E restricted cells were present , confirming this hypothesis. However, at high infective doses the response of resistant strains of mice were suppressed to a point at which the responses of I-E^+ and I-E^- were similar. In order to explain this phenomenon it was proposed that I-A restricted cells could recognise parasite antigens with high affinity and mediate a strong response at low doses. At high doses this response could be down regulated when the number of activated T cells increased in proportion to the numbers of auto-reactive regulatory cells. In contrast, I-E restricted cells would induce auto-reactive cells which would down regulate the otherwise strong I-A response.

I-E^+ mice are thought to be actively immunosuppressed by a mechanism involving the induction of I-J^+ suppressor cells. I-J molecules are non-H-2 but their expression is regulated by I-region genes. It is thought that I-J^k expression requires

complementation from at least 2 other genes. I-J^+ T cell receptors are expressed on the surface of suppressor and contrasuppressor cells which regulate T helper activity. Thus, mice are thought to be resistant to this infection either due to a lack of the I-E molecule or the presence of an improper allele for I-J^k expression at the relevant loci mapping outside the MHC. As it is thought that the parasite actively produces a factor which is able to suppress the hosts immune response (Pritchard, Ali and Behnke, 1984) it was further suggested that the factor was presented in the context of I-E molecules and/or that the I-J molecule recognises it thus inducing further suppression of the immune response.

In summary, it appears that the genotype of the host influences its ability to overcome the parasite immunomodulatory factor and expel the parasite and that the immune response was generated and effective against the adult stage of the parasite rather than the larval stages as has been demonstrated for a number of immunising regimes (Behnke and Robinson, 1985; Behnke and Wakelin, 1977; Enriquez, Cypess and Wassom, 1988; Ey, 1988a,b; Goven and De Buysscher, 1980; Hagan, Behnke and Parish, 1981; Hosier, Sackman and Idell, 1974; Jones and Rubin, 1974; Mitchell and Munoz, 1983; van Zandt, 1973).

CHAPTER SIX

INFLUENCE OF MOUSE STRAIN ON THE IMMUNOLOGICAL RESPONSE TO AN IMMUNISING INFECTION OF *HELIGMOSOMOIDES POLYGYRUS*

6.1 INTRODUCTION

A variety of immunising schedules have been devised by a number of authors. They provide different levels of immunity and are dependent on the strain of mouse host used. Some involve vaccination with whole larvae or their extracts, infection with irradiated larvae or multiple infections with normal larvae terminated with anthelmintics.

It was decided to use the 9-day anthelmintic abbreviated infection devised by Behnke and Robinson (1985). This protocol allows the synchronised normal development of the third and fourth larval stages, but the anthelmintic treatment from day 9 onwards removes the adult parasites from the gut lumen as they emerge from the submucosa without affecting parasites which are still developing in the tissues. This regime has been found to be very effective in discriminating between weak and strong responder strains of the mouse host. It is thought that this protocol elicits a strong anti-larval stage specific immunity as transplanted adult parasites are not expelled from immunised hosts (Robinson, Behnke and Williams, 1988). It has been proposed that *H. polygyrus* can evade host protective responses, however, this is not always the case and the parasite is not always successful. Different strains of mice appear to express a genetically determined susceptibility to the parasite mediated immunomodulation which results in the inability of some strains to expel the parasite despite the immunising regime being successful in other strains of the host.

The purpose of this study was to try to analyse the mechanisms which promoted the generation of an immune response against the parasite and to investigate factors which may be responsible for the generation of resistance in some strains whilst other strains remain susceptible to challenge infections. This experiment allowed the monitoring of all the events accompanying immunity to the parasite and the times at which they occurred.

The experiment was designed thus, for each strain of the mouse host used, one group, B, was immunised using the 9-day abbreviated schedule. Briefly, the mice were infected 200 larvae, 9 days later they were dosed with 100mg/kg of pyrantel embonate (Pfizer), this was repeated on days 14 and 21. This dose of the anthelmintic has been shown to be 100% effective at removing adult worms worms from the intestinal lumen but leaves developing larvae unaffected (Behnke and Wakelin, 1977). This group was then challenged with 100 infective larvae 28 days after the initial infection. Half of this group was then killed on day 28 (before challenge), and half on day 42 (after challenge). This period after challenge was chosen so that the infectivity of the challenge infection could be assessed. Challenge controls were infected with 100 L3 on day 28 and half sacrificed on day 42 and half when the parasites had been expelled (week 6 for SJL, week 13 for BALB/c and week 35 for CBA).

Group C were infected with 200 larvae on day 0 and 28 days later were superimposed with 100 larvae. Group were then killed on day 21, in order to assess the infectivity of the larvae. On day 42, 14 days after challenge the next group was autopsied and then subsequently when the parasite was eventually expelled from the host as assessed by egg counts as for the previous experiment in Chapter 5. For SJL, this was 9 weeks p.i., for BALB/c, this was 31 weeks p.i. and for CBA, this was 41 weeks p.i..

CBA was also represented by a further group, D, as it was known (Behnke and Robinson, 1985) that the immunising protocol used does not result in resistance to reinfection by the parasite in this strain and so it was thought that the longevity and/or the fecundity of the parasite may be affected by an immune response. Thus, this group was immunised and challenged as for group B, but the mice were not killed until the parasite had been expelled.

Each group of animals killed was represented by 4 animals. Egg counts were performed weekly, and WBC counts, blood smears and sera was taken fortnightly. At each autopsy a

number of immunological parameters were investigated including cellularity of the secondary lymphoid organs, enumeration of T and B cells in these organs and the responsiveness of the cells to mitogens and enumeration of mucosal mast cells in the gut.

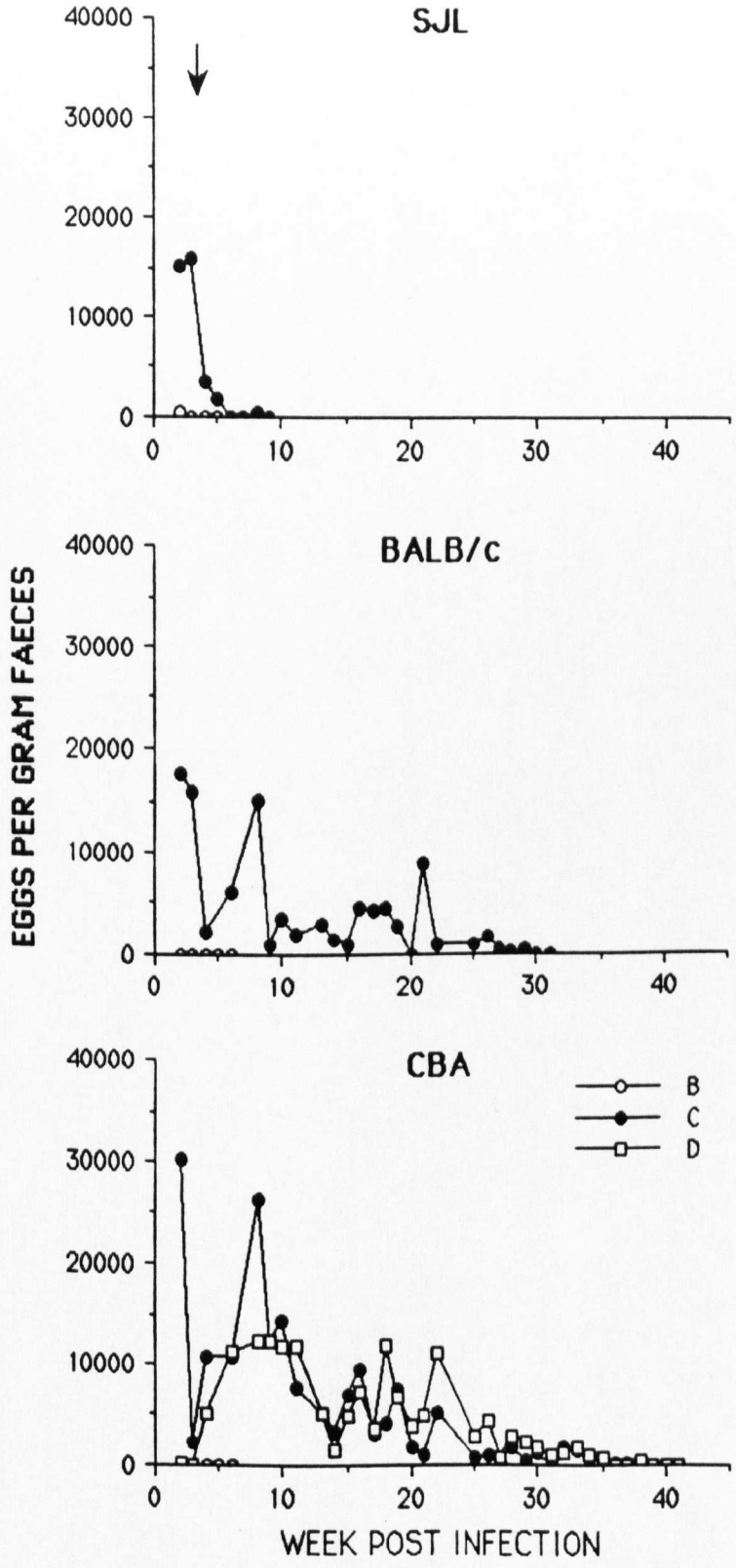
6.2 RESULTS

6.2.1 Faecal Egg Counts

Fig. 6.1 shows the faecal egg counts of all the groups. It was apparent that in all the strains immunised, group B, no eggs were produced during the time course of the experiment. In groups C and D, when the challenge infection was given, the egg counts were severely reduced when compared to a primary infection of a similar intensity. In BALB/c and SJL, there was no difference between the egg counts whilst those of CBA were slightly higher, however, the counts in group D, the CBA immunised group, were markedly reduced.

Figure 6.1 Faecal Egg Counts

Faecal egg counts were performed weekly by standard zinc sulphate flotation for SJL, BALB/c and CBA mice immunised (B and D) and unimmunised (C) groups. The point of challenge is shown with the arrow.


SJL
BALB/c
CBA
B
C
D
40000
30000
20000
10000
0
0
10
20
30
40
EGGS PER GRAM FAECES
WEEK POST INFECTION

6.2.2 Worm Burdens

When the worm burdens of the various groups were assessed (Fig. 6.2), initially the infection in all groups was slightly lower than in a primary infection. Group B of BALB/c and CBA had fewer worms after challenge than the unimmunised group C or the challenge control, however, those of SJL were not different from the unimmunised group but were lower than the challenge controls.

Figure 6.2 Worm Burdens

The parasite burdens of SJL, BALB/C and CBA mice immunised (B and D) and unimmunised (C) groups were assessed at various time points. The mean and the standard error for each time point was calculated. The point of challenge is represented by an arrow.

SJL

C3 vs B6: $p < 0.05$ C3 vs C6: $p < 0.05$ C6 vs B6: NS

BALB/c

C3 vs B6: NS C3 vs C6: NS C3 vs C31: NS

C6 vs B6: NS C6 vs C31: NS

CBA

C3 vs B6: $p < 0.05$ C3 vs C6: $p < 0.05$

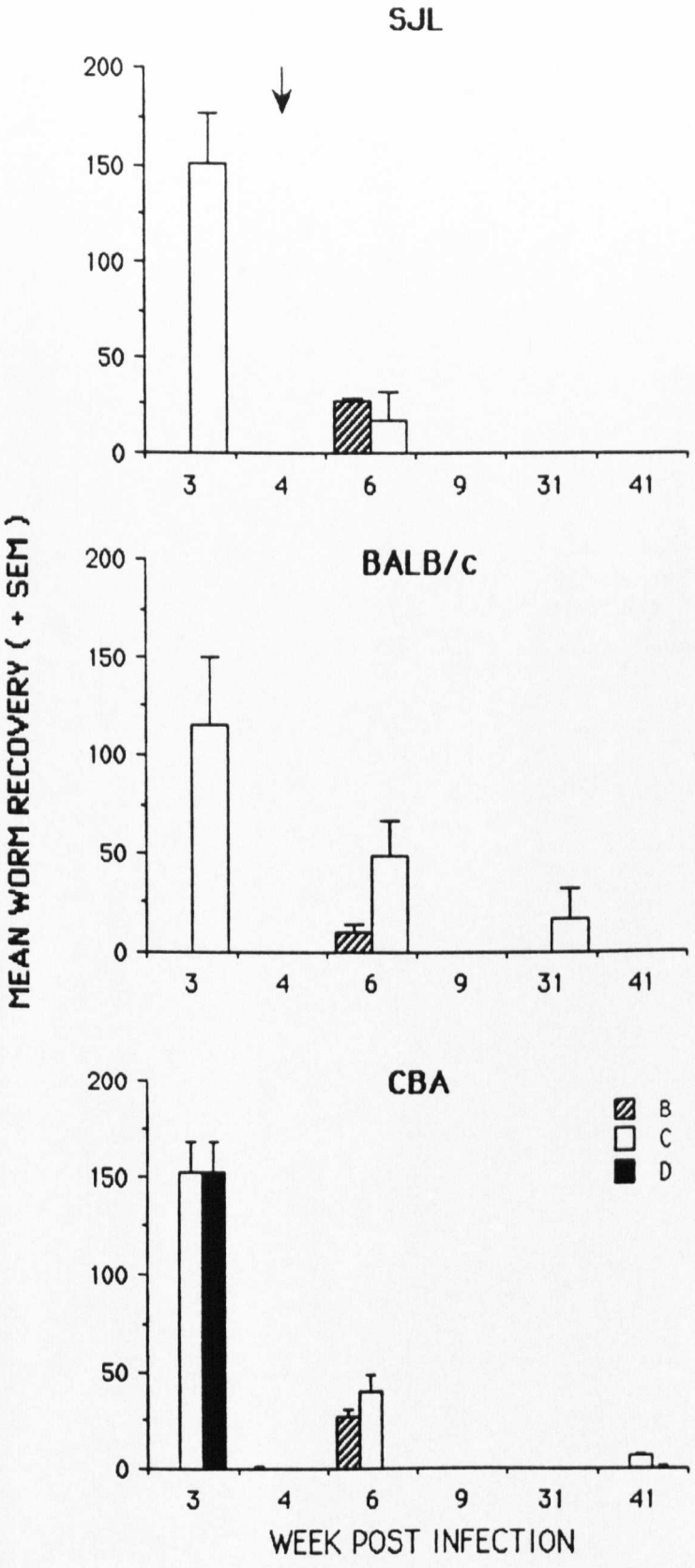
C3 vs C41: $p < 0.05$ B6 vs B41: $p < 0.05$

SJL B6 vs BALB/c B6: $p < 0.05$ SJL B6 vs CBA B6: NS

CBA B6 vs BALB/c B6: NS SJL C6 vs BALB/c C6: NS

SJL C6 vs CBA C6: NS

N.B. NS (not significant)


SJL
BALB/c
CBA
MEAN WORM RECOVERY (+ SEM)
200
150
100
50
0
3
4
6
9
31
41
B
C
D
WEEK POST INFECTION

6.2.3 White Blood Cell Counts

Fig. 6.3 indicates that there was a rise in the peripheral WBC counts in all of the groups following infection. In all of the strains, group B rose in response to the challenge, with SJL rising higher than BALB/c or CBA. In CBA and BALB/c there was no apparent increase in the cellularity of groups C or D. However, in SJL, there was a sharp rise 2 weeks after challenge, in the unimmunised group followed by a decline until the parasite was expelled.

Figure 6.3 White Blood Cell Counts

The numbers of white blood cells present in the blood of immunised (B and D) and unimmunised (C) groups of SJL, BALB/c and CBA mice was measured fortnightly. The mean and standard error of the number of cells present per ul of blood was calculated. The point of challenge is represented by an arrow.

SJL

B0 vs B4: NS B0 vs B6: $p < 0.05$ B4 vs B6: NS

C0 vs C6: NS C0 vs C9: NS C6 vs B6: NS

BALB/c

B0 vs B4: NS B0 vs B6: NS B4 vs B6: NS

C0 vs C6: NS C0 vs C31: NS C6 vs B6: NS

CBA

B0 vs B4: NS B0 vs B6: $p < 0.05$ D0 vs D41: NS

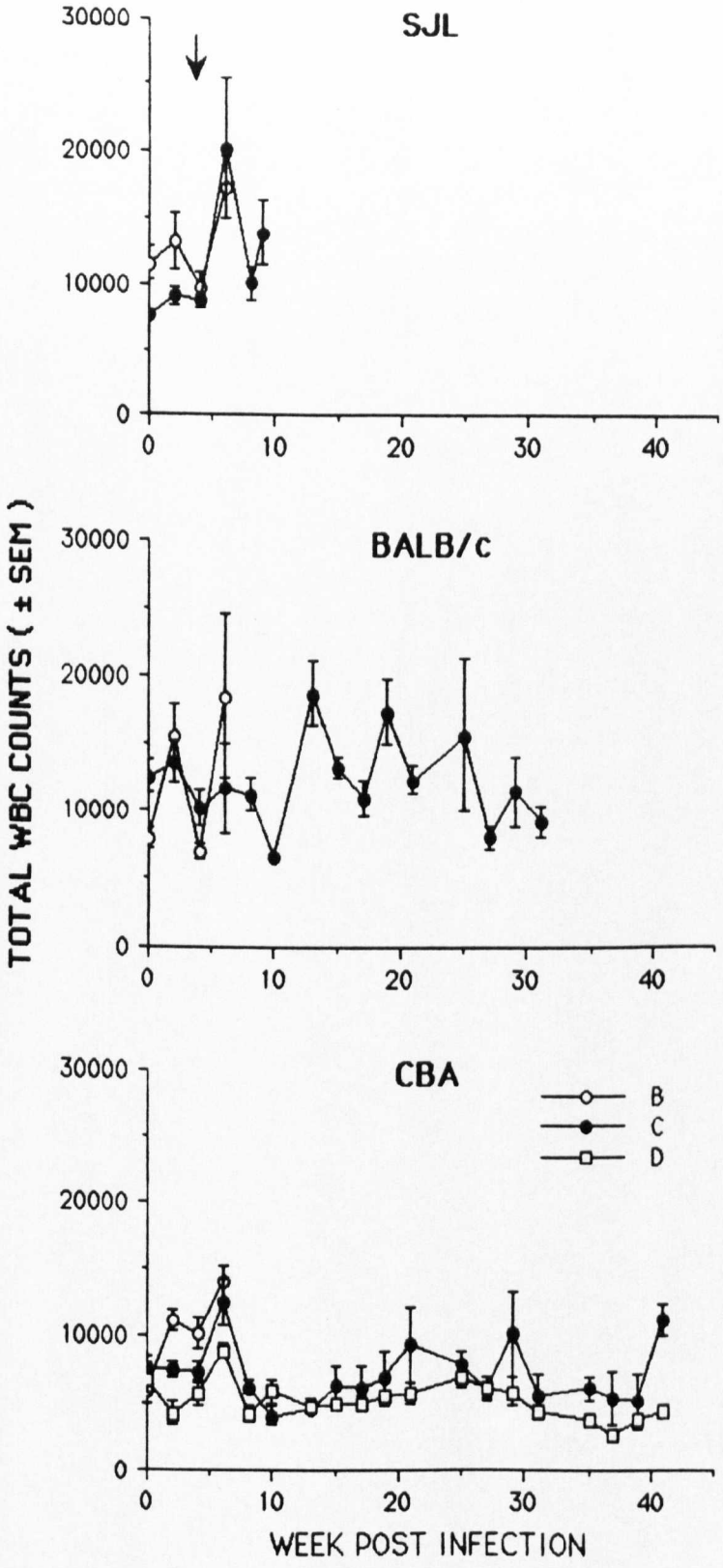
C0 vs C6: $p < 0.05$ C0 vs C41: $p < 0.05$ C6 vs B6: NS

SJL C6 vs CBA C6: NS SJL B6 vs CBA B6: NS

SJL C9 vs BALB/c C31: NS SJL C9 vs CBA C41: NS

SJL C9 vs CBA D41: $p < 0.05$ CBA D41 vs BALB/c C31: $p < 0.05$

N.B. NS (not significant)


SJL
BALB/c
CBA
B
C
D
30000
20000
10000
0
0
10
20
30
40
TOTAL WBC COUNTS (± SEM)
WEEK POST INFECTION

6.2.4 Differential Cell Counts

When the differential cell populations were analysed it was noted (Fig. 6.4) that in all strains and groups there was no significant change in the percentage of eosinophils although a slight rise was apparent in both groups of BALB/c. In groups C and D of all strains the percentage of lymphocytes appeared to fall at challenge but then rose until expulsion of the worm occurred.

Figure 6.4 Percentage of Lymphocytes and Eosinophils

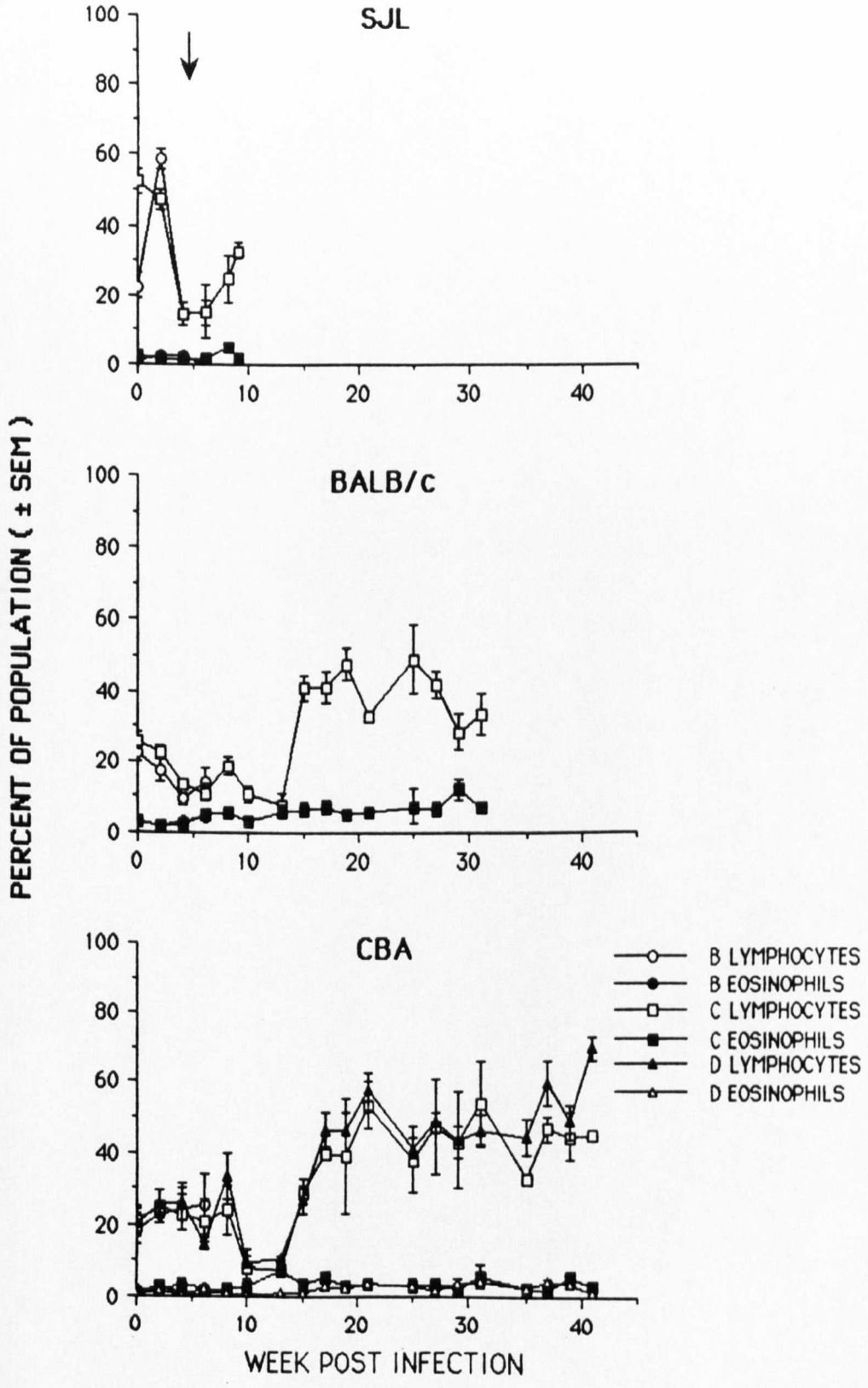
The percentages of lymphocytes and eosinophils present was measured fortnightly from Giemsa stained blood smears taken from immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice. The mean and standard error was calculated and the time of challenge is indicated by an arrow.

Lymphocytes
SJL
B0 vs B6: NS C0 vs C6: NS C0 vs C9: NS C6 vs C9: NS
BALB/c
B0 vs B6: NS C0 vs C6: $p < 0.05$ C0 vs C31: NS
C6 vs C31: $p < 0.05$
CBA
B0 vs B6: NS C0 vs C6: NS C0 vs C41: $p < 0.05$
C6 vs C41: $p < 0.05$ D0 vs D41: $p < 0.05$ D6 vs D41: $p < 0.05$

SJL B6 vs BALB/c B6: NS SJL B6 vs CBA B6: NS
CBA B6 vs BALB/c B6: NS SJL C6 vs CBA C6: NS
CBA C6 vs BALB/c C6: NS SJL C9 vs BALB/c C31: NS
SJL C9 vs CBA C41: $p < 0.05$ BALB/c C31 vs CBA C41: NS

Eosinophils
SJL
B0 vs B6: NS C0 vs C6: NS C0 vs C9: NS C6 vs C9: NS
BALB/c
B0 vs B6: NS C0 vs C6: NS C0 vs C31: NS C6 vs C31: NS
CBA
B0 vs B6: NS C0 vs C6: NS C0 vs C41: NS D0 vs D41: NS
C6 vs C41: $p < 0.05$ D6 vs D41: NS

SJL B6 vs BALB/c B6: $p < 0.05$ SJL B6 vs CBA B6: NS
CBA B6 vs BALB/c B6: NS SJL C6 vs CBA C6: NS
SJL C6 vs BALB/c C6: $p < 0.05$ CBA C6 vs BALB/c C6: $p < 0.01$
SJL C9 vs BALB/ c C31: NS SJL C9 vs CBA C41: NS
CBA C41 vs BALB/c C31: NS


SJL
BALB/c
CBA
PERCENT OF POPULATION (± SEM)
WEEK POST INFECTION
B LYMPHOCYTES
B EOSINOPHILS
C LYMPHOCYTES
C EOSINOPHILS
D LYMPHOCYTES
D EOSINOPHILS

Examination of the percentage of monocytes (Fig. 6.5) revealed a slight rise in group B of all strains and in group C of SJL, but there was no apparent change in the remaining groups. The percentage of neutrophils increased in all groups of BALB/c and CBA but did not appear to alter appreciably in SJL.

Figure 6.5 Percentage of Neutrophils and Monocytes

The percentages of monocytes and neutrophils present was measured fortnightly from Giemsa stained blood smears taken from immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice. The mean and standard error was calculated and the time of challenge is indicated by an arrow.

Neutrophils

SJL

B0 vs B6: NS C0 vs C6: NS C6 vs C9: NS C0 vs C9: $p < 0.05$

BALB/c

B0 vs B6: NS C0 vs C6: NS C0 vs C31: NS C6 vs C31: NS

CBA

B0 vs B6: NS C0 vs C6: NS C0 vs C41: NS C6 vs C41: NS

D0 vs D41: NS D6 vs D41: $p < 0.05$

SJL B6 vs BALB/c B6: NS
SJL B6 vs CBA B6: NS
CBA B6 vs BALB/c B6: NS
SJL C6 vs BALB/c C6: $p < 0.05$
SJL C6 vs CBA C6: $p < 0.05$
CBA C6 vs BALB/c C6: NS
CBA C41 vs BALB/c C31: NS
SJL C9 vs BALB/c C31: $p < 0.05$
SJL C9 vs CBA C41: $p < 0.05$

Monocytes

SJL

B0 vs B6: NS C0 vs C6: NS C0 vs C9: NS C6 vs C9: NS

BALB/c

B0 vs B6: $p < 0.05$ C0 vs C6: NS C0 vs C31: NS

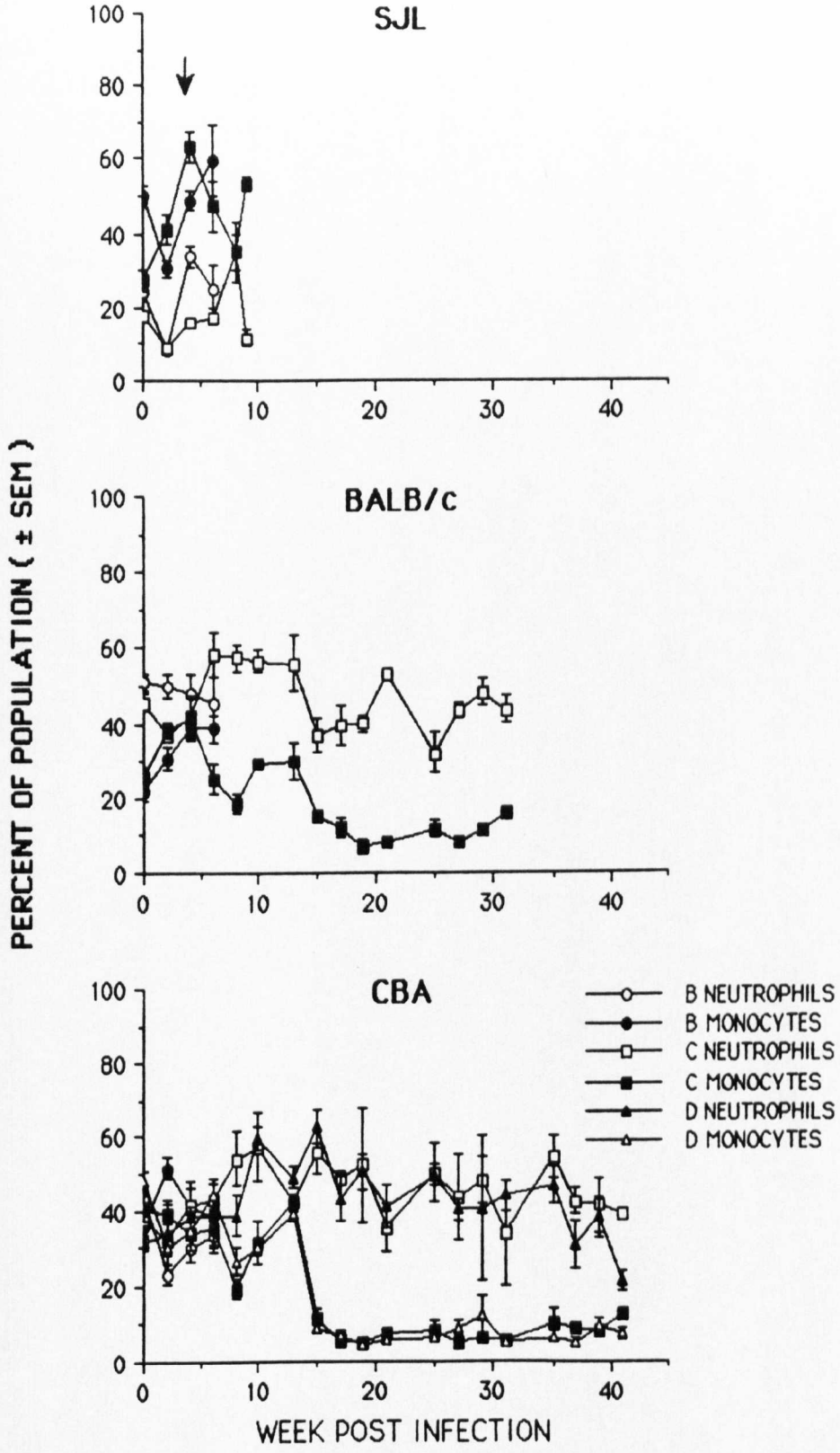
C6 vs C31: $p < 0.05$

CBA

B0 vs B6: NS C0 vs C6: NS C0 vs C41: $p < 0.05$

C6 vs C41: $p < 0.05$ D0 vs D41: $p < 0.05$ D6 vs D41: $p < 0.05$

SJL B6 vs BALB/c B6: NS
SJL B6 vs CBA B6: NS
CBA B6 vs BALB/c B6: NS
SJL C6 vs BALB/c C6: $p < 0.05$
SJL C6 vs CBA C6: NS
CBA C6 vs BALB/c C6: NS
CBA C41 vs BALB/c C31: NS
SJL C9 vs CBA C41: $p < 0.005$


SJL
BALB/c
CBA
PERCENT OF POPULATION (± SEM)
WEEK POST INFECTION
B NEUTROPHILS
B MONOCYTES
C NEUTROPHILS
C MONOCYTES
D NEUTROPHILS
D MONOCYTES
0
20
40
60
80
100
0
10
20
30
40

When the total numbers of these cells types were calculated (Fig. 6.6) it was again apparent that there was little or no eosinophilia in SJL or CBA although a slight rise was obvious in BALB/c. In group B, the numbers of lymphocytes in BALB/c and SJL rose after infection, dropped as the worms were removed with anthelmintic and then rose again after challenge. CBA rose for the whole experiment although the numbers were lower than in the other two groups. In group C, the unimmunised group, this pattern was mirrored except that the rise was prolonged until the parasite was expelled.

Figure 6.6 Total Numbers of Lymphocytes and Eosinophils

The total numbers of lymphocytes and eosinophils present per ul blood of immunised (B and D) and unimmunised (C) groups of SJL, BALB/c and CBA mice was calculated from the figures obtained from the total white blood cell counts and the percentage of the cell population present. The mean and standard error were calculated and the point of challenge is represented by an arrow.

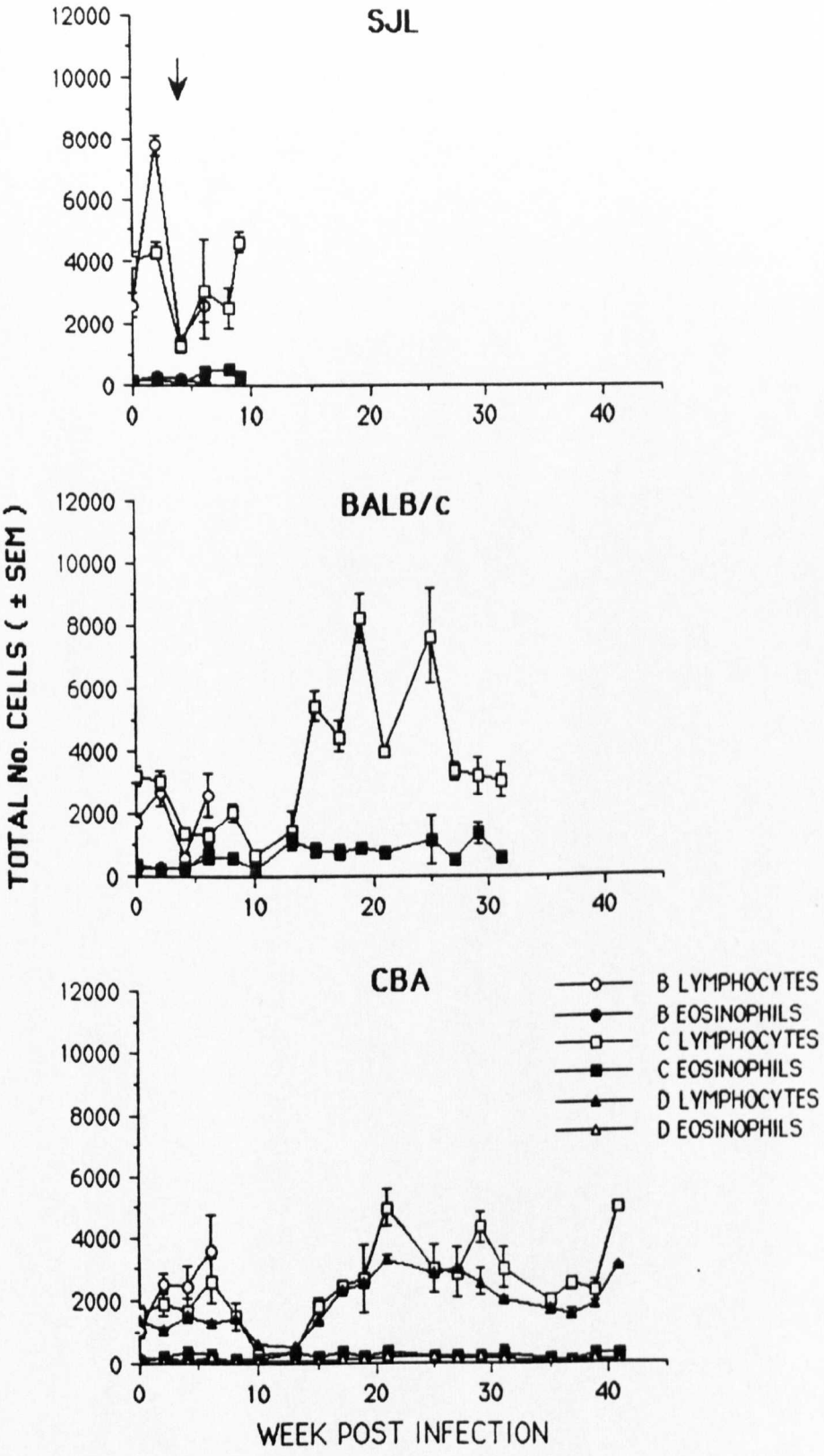
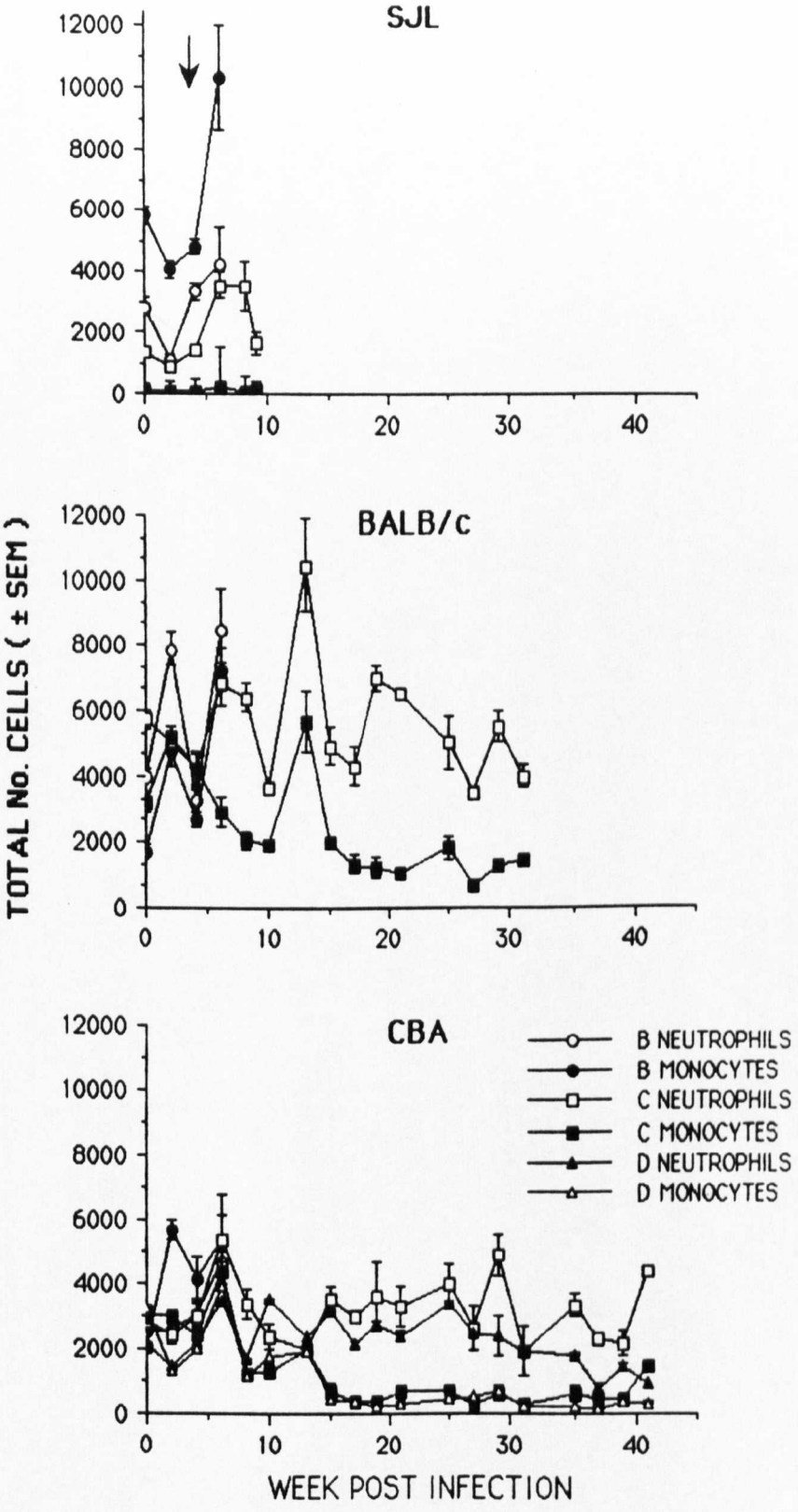

SJL
BALB/c
CBA
TOTAL No. CELLS (± SEM)
WEEK POST INFECTION
B LYMPHOCYTES
B EOSINOPHILS
C LYMPHOCYTES
C EOSINOPHILS
D LYMPHOCYTES
D EOSINOPHILS
0
2000
4000
6000
8000
10000
12000
0
10
20
30
40

Fig. 6.7 shows the total numbers of monocytes and neutrophils. In SJL B there was a dramatic increase in the numbers of both monocytes and neutrophils after challenge, however, the numbers of these cells in SJL C, the unimmunised group, were negligible in comparison. In BALB/c and CBA the picture was slightly different, in all groups there was a rise in the numbers of neutrophils corresponding with a drop in the numbers of monocytes.

Figure 6.7 Total Numbers of Monocytes and Neutrophils

The total numbers of monocytes and neutrophils present per ul blood of immunised (B and D) and unimmunised (C) groups of SJL, BALB/c and CBA mice was calculated from the figures obtained from the total white blood cell counts and the percentage of the cell population present. The mean and standard error were calculated and the point of challenge is represented by an arrow.

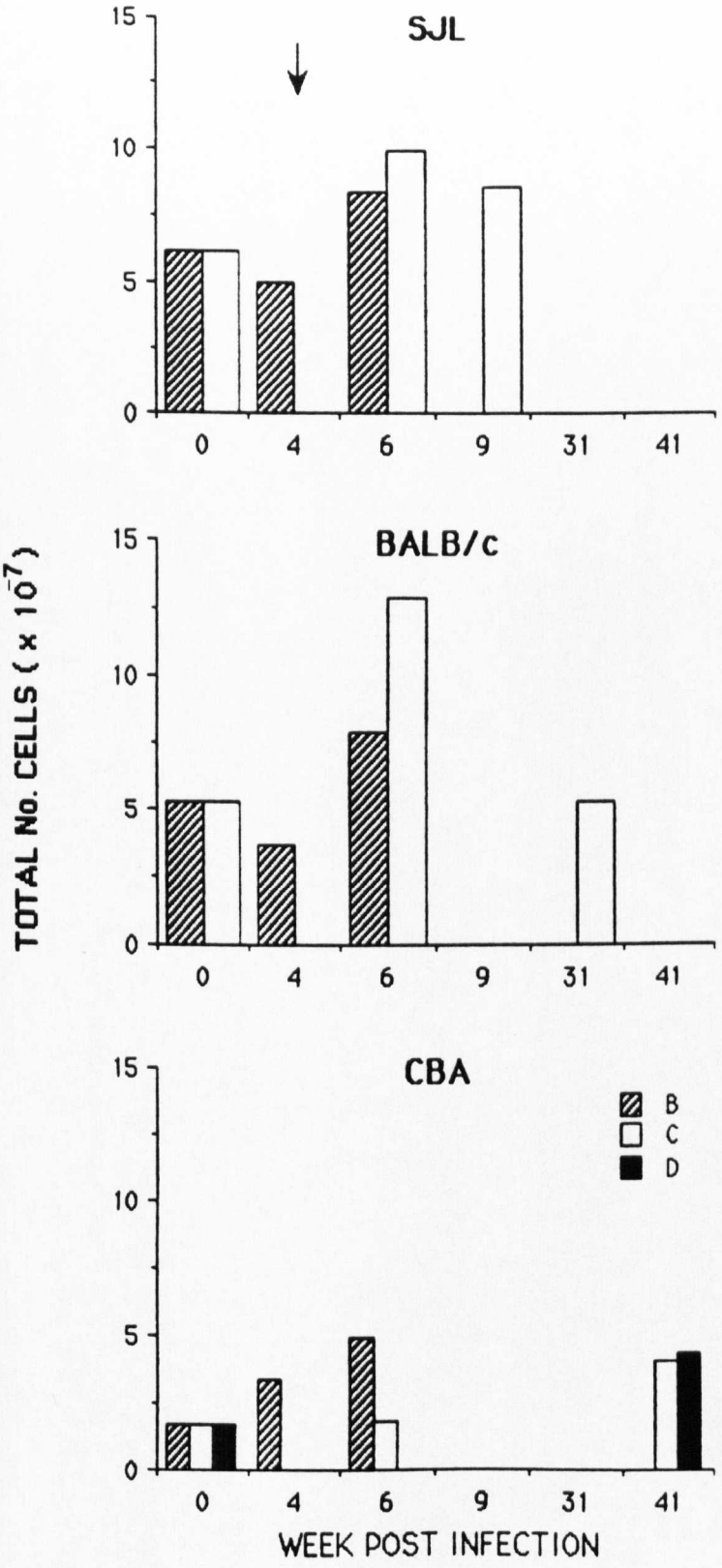

SJL
BALB/c
CBA
TOTAL No. CELLS (± SEM)
WEEK POST INFECTION
B NEUTROPHILS
B MONOCYTES
C NEUTROPHILS
C MONOCYTES
D NEUTROPHILS
D MONOCYTES
0
2000
4000
6000
8000
10000
12000
0
10
20
30
40

6.2.5 Cellular Response of the Secondary Lymphoid Organs

Next, the secondary lymphoid organs, namely, the spleen and mesenteric lymph nodes, were examined. Unfortunately, it was necessary to use pooled cells from the secondary lymphoid organs for each group of animals, because if the organs were to be prepared separately, the cells would have died before they could be analysed. Thus, no statistical analysis can be performed on this data. Fig. 6.8 shows the total number of viable cells in the spleen. The cellularity of the spleen in group B of all the strains rose in response to the challenge infection. That of group C, the unimmunised group, also rose but fell after challenge as the parasite was being expelled. In each case SJL had a greater cellularity than BALB/c which, in turn, was larger than CBA.

Figure 6.8 Total Cellularity of the Spleen

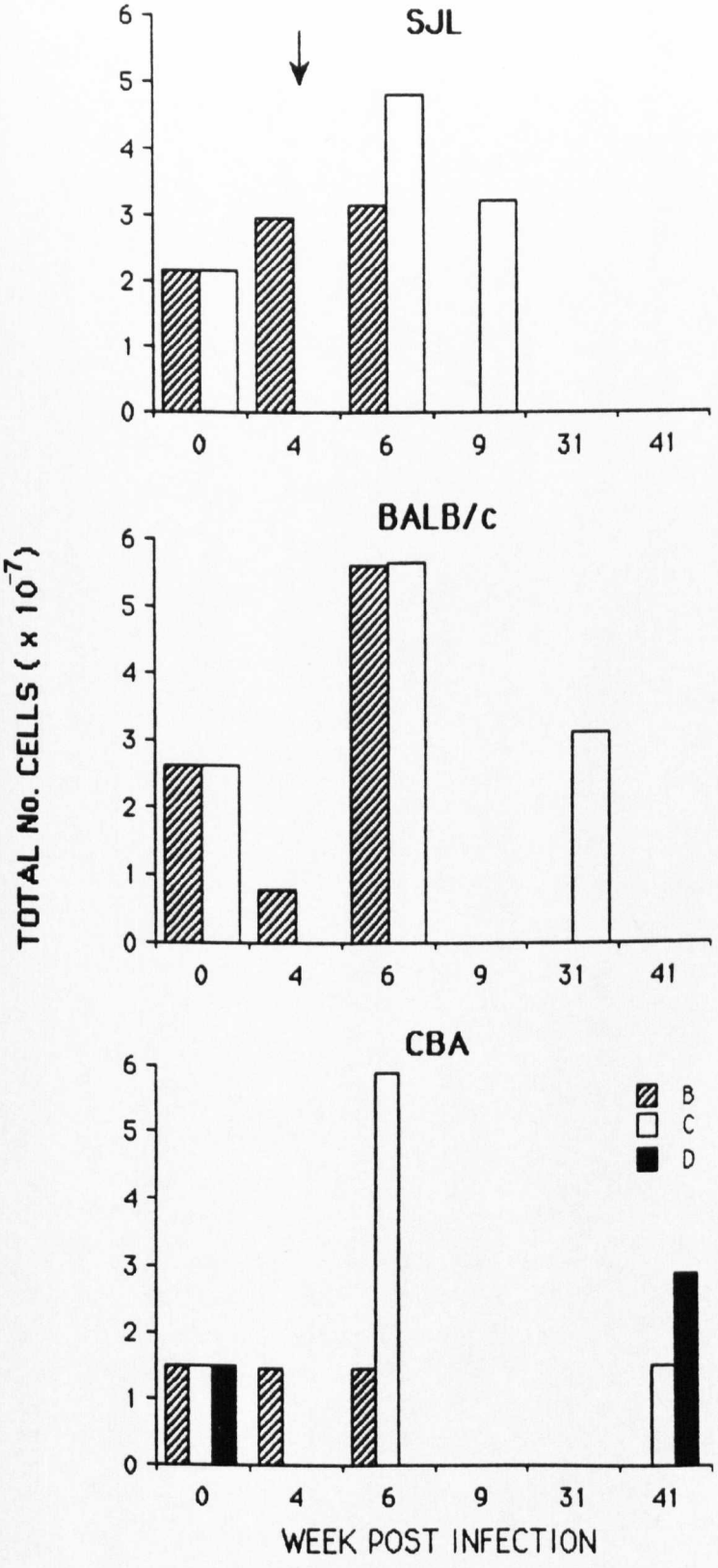
The total numbers of viable cells present in the spleen of immunised (B and D) and unimmunised animals (C) was calculated at each autopsy by assessing their ability to convert fluorescein diacetate to free fluorescein. Pooled cells were used in the study and the numbers of cells per mouse was calculated, however, the standard error could not be calculated. The time of challenge is shown by the arrow.


SJL
BALB/c
CBA
B
C
D
15
10
5
0
0
4
6
9
31
41
TOTAL No. CELLS (x 10^{-7})
WEEK POST INFECTION

The cellularity of the MLN (Fig. 6.9) revealed that there was little change in group B although a slight increase was apparent in BALB/c. Again, the response of group C increased after challenge but fell when the parasite was expelled.

Figure 6.9 Total Cellularity of the Mesenteric Lymph Nodes

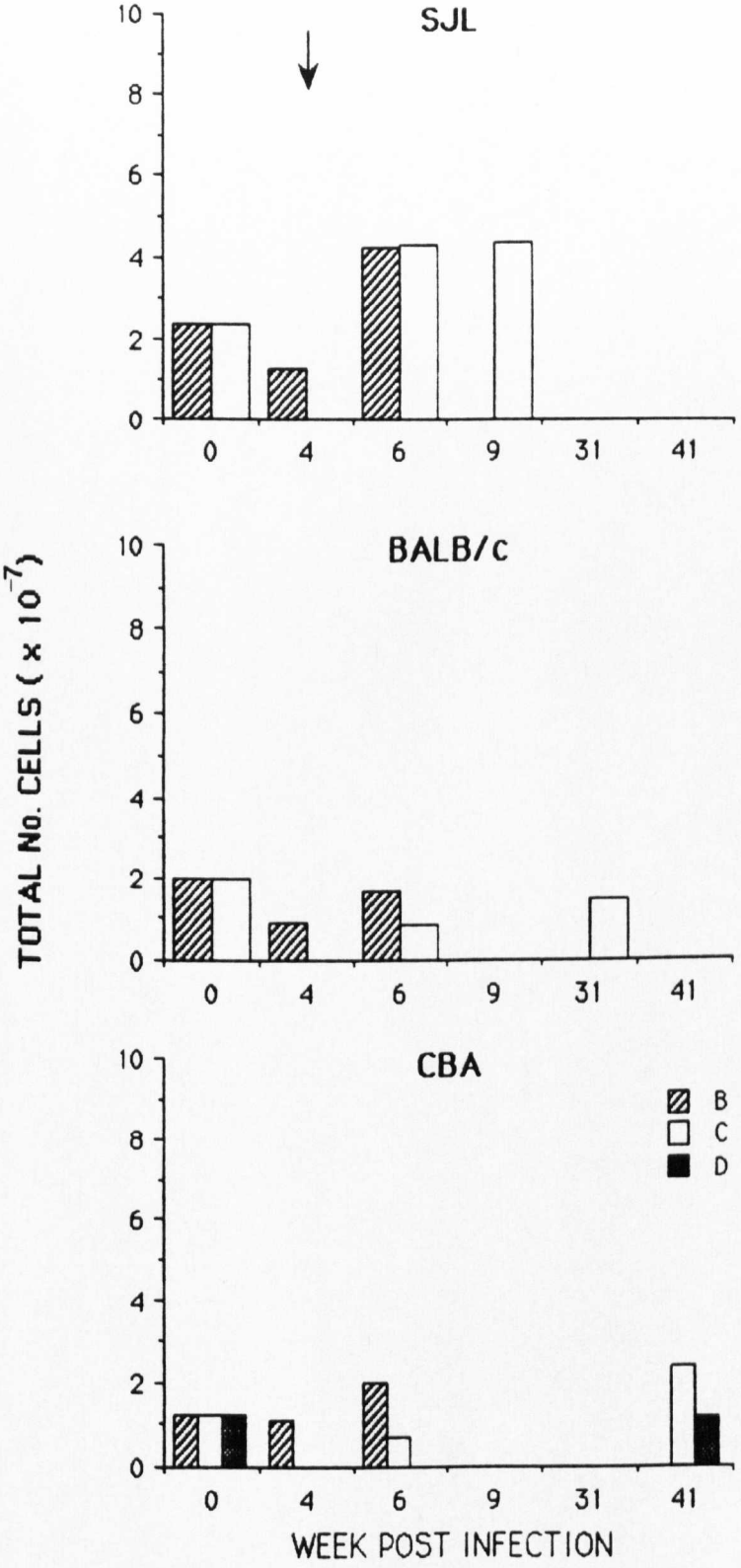
The total numbers of viable cells present in the MLN of immunised (B and D) and unimmunised animals (C) was calculated at each autopsy by assessing their ability to convert fluorescein diacetate to free fluorescein. Pooled cells were used in the study and the numbers of cells per mouse was calculated, however, the standard error could not be calculated. The time of challenge is shown by the arrow.


SJL
BALB/c
CBA
TOTAL No. CELLS (x 10^{-7})
0
1
2
3
4
5
6
0
4
6
9
31
41
B
C
D
WEEK POST INFECTION

A more detailed analysis of the events occurring within these organs was initiated by enumerating the viable T, B and null cell populations. Fig. 6.10 illustrates the numbers of B cells in the spleen. It was apparent that there was a similar increase in the numbers of these cells in both groups of SJL. In BALB/c there was no significant increase in cellularity in any of the groups and there was even a slight drop in numbers. In CBA, again, there was little change in any of the groups.

Figure 6.10 Numbers of B Cells in the Spleen

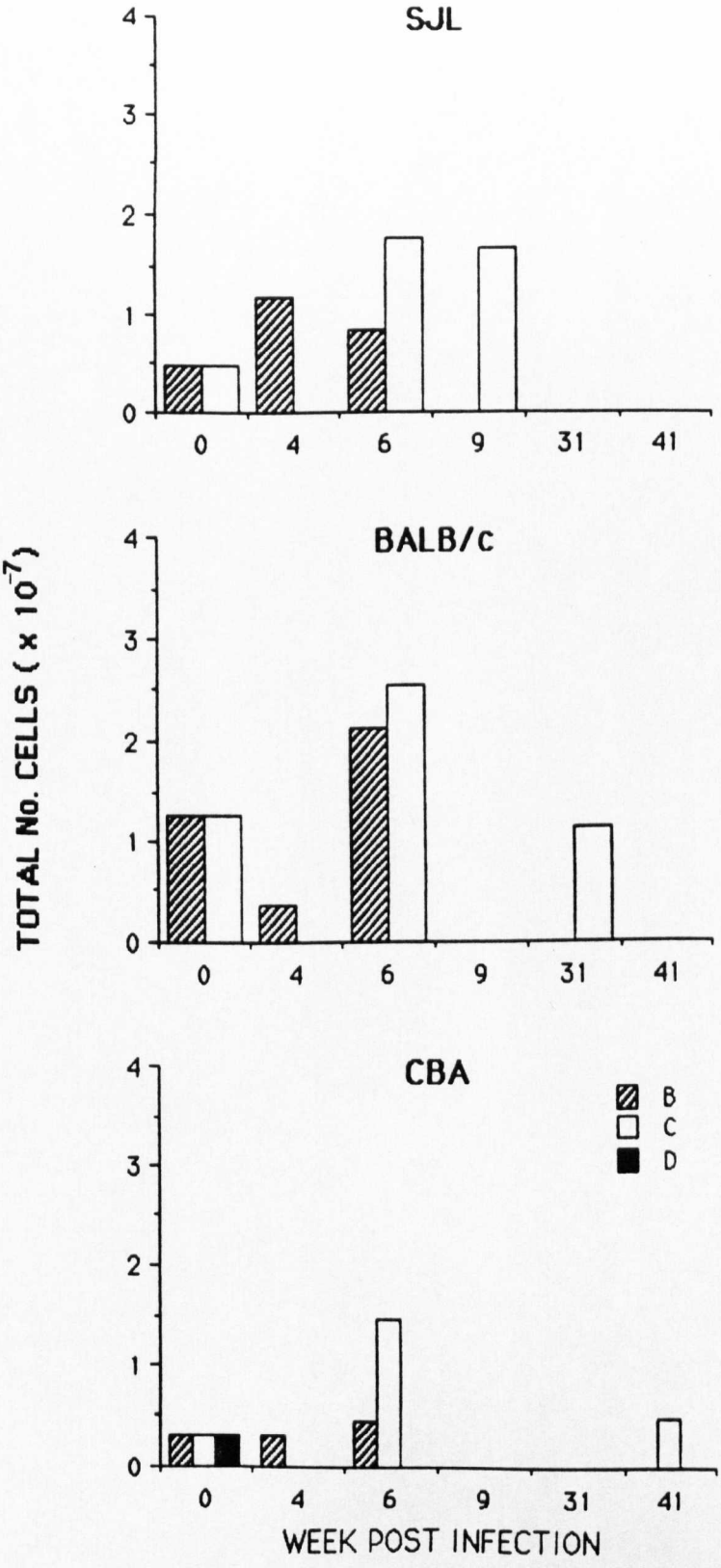
The total numbers of viable B cells present in the spleens of immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice was calculated by using an FITC-conjugated antibody directed against mouse IgG,A and M. The time of challenge is indicated by the arrow.


SJL
BALB/c
CBA
B
C
D
TOTAL No. CELLS (x 10^{-7})
WEEK POST INFECTION
0
2
4
6
8
10
0
4
6
9
31
41

When the MLN were examined (Fig. 6.11) there was no difference in groups B and D of all the strains, although a slight rise was visible in the numbers of B cells in the immunised group, B.

Figure 6.11 Numbers of B Cells in the Mesenteric Lymph Nodes

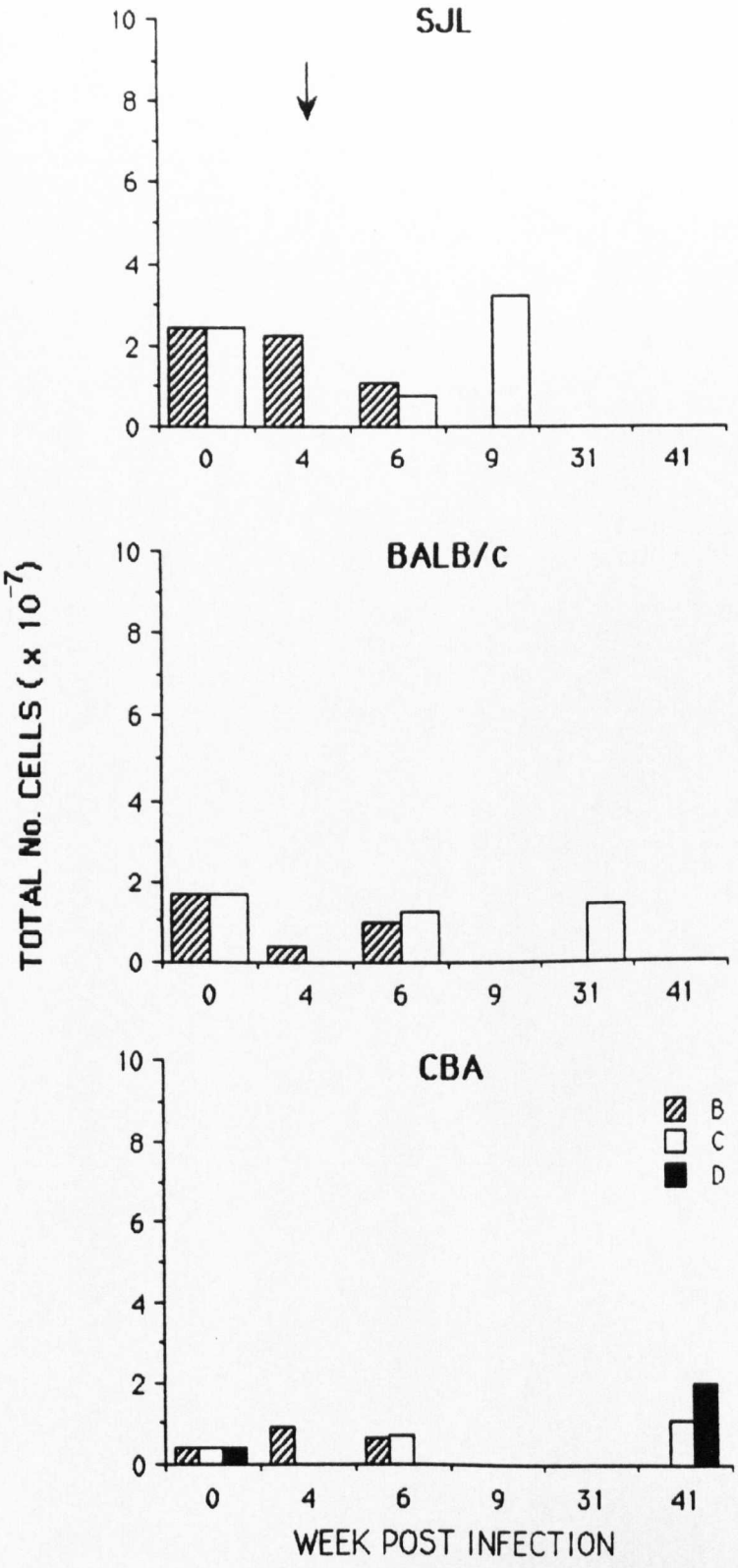
The total numbers of viable B cells present in the MLN of immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice was calculated by using an FITC-conjugated antibody directed against mouse IgG,A and M. The time of challenge is indicated by the arrow.


SJL
BALB/c
CBA
B
C
D
TOTAL No. CELLS (x 10^{-7})
0
1
2
3
4
0
4
6
9
31
41
WEEK POST INFECTION

The numbers of viable T cells present in the spleen are represented in Fig. 6.12, there was no appreciable change in the numbers of these cells in the spleens of any of the groups or strains.

Figure 6.12 Numbers of T cells in the Spleen

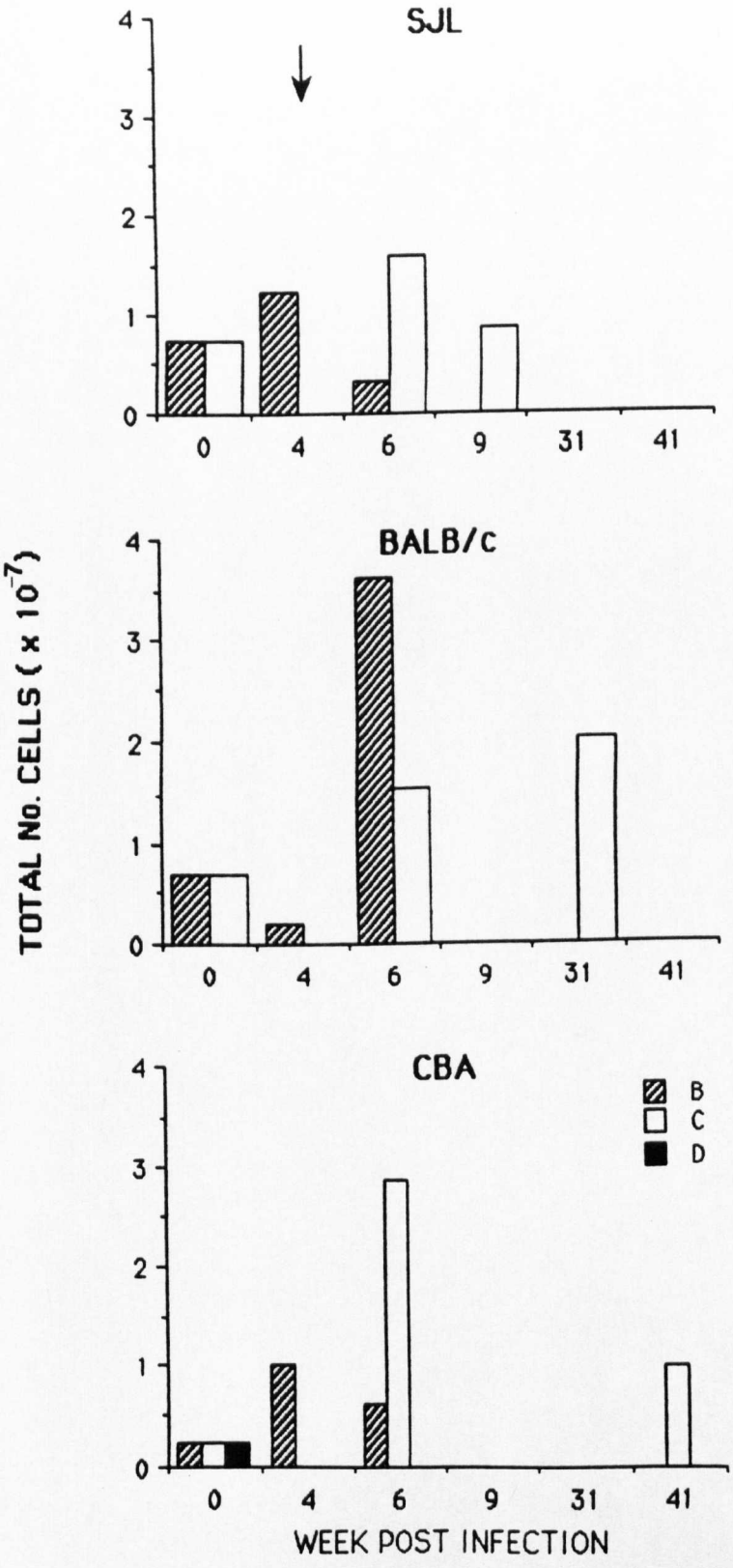
The total numbers of viable T cells present in the spleens of immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice was calculated by using an FITC-conjugated antibody directed against mouse Thy.1. The time of challenge is indicated by the arrow.


SJL
BALB/c
CBA
TOTAL No. CELLS (x 10⁻⁷)
WEEK POST INFECTION
B
C
D
0
2
4
6
8
10
0
4
6
9
31
41

In the MLN (Fig. 6.13), however, the unimmunised group, C, showed a rise in all strains whilst in group B the greatest increase was in BALB/c.

Figure 6.13 Numbers of T cells in the Mesenteric Lymph Nodes

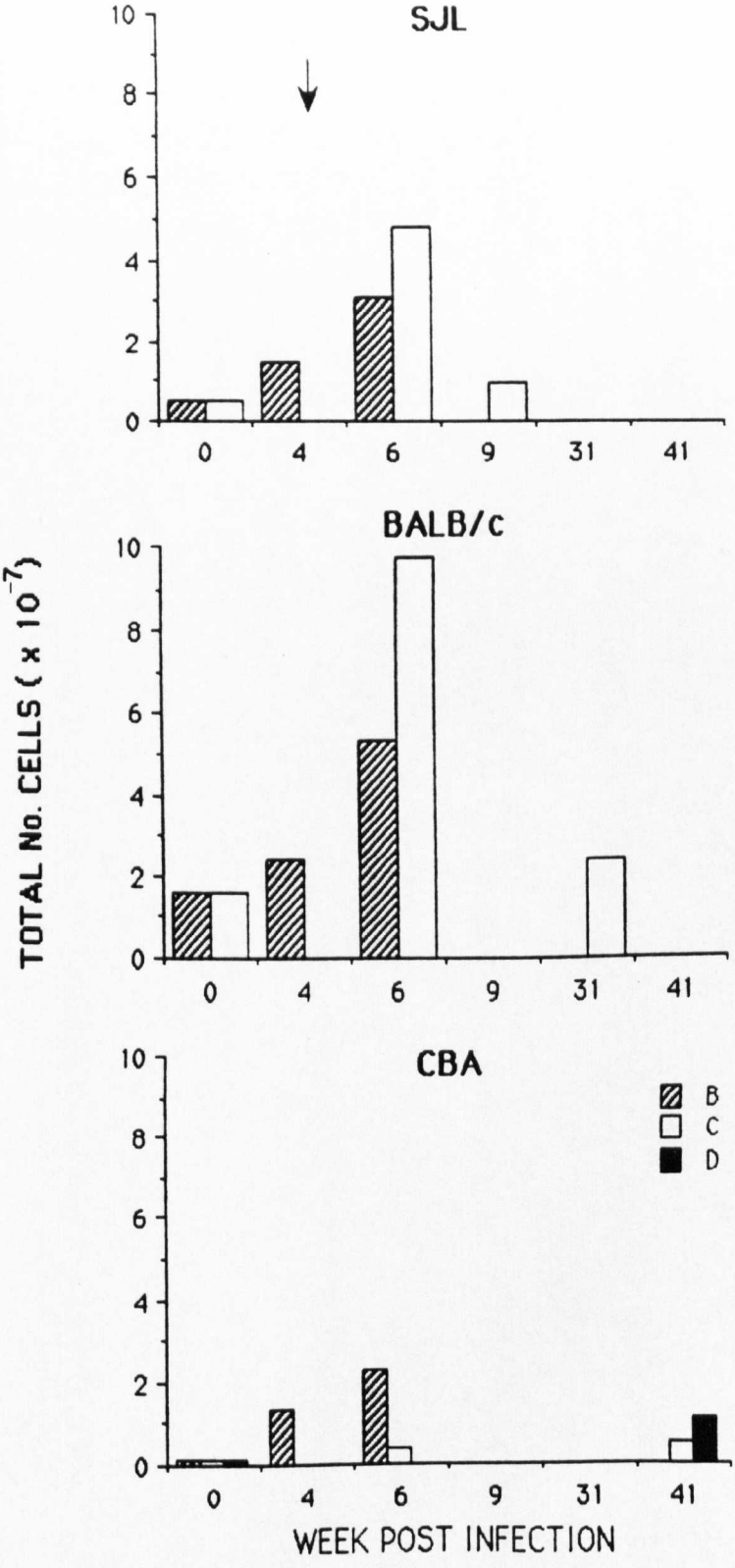
The total numbers of viable T cells present in the MLN of immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice was calculated by using an FITC-conjugated antibody directed against mouse Thy.1. The time of challenge is indicated by the arrow.


SJL
BALB/c
CBA
B
C
D
TOTAL No. CELLS (x 10^{-7})
0
1
2
3
4
0
4
6
9
31
41
WEEK POST INFECTION

When the numbers of viable null cells (non T/B cells) were calculated, the spleen (Fig. 6.14) showed an increase in cellularity in group B whereas group C rose at challenge but then declined until the parasite was rejected. The changes were most marked in SJL followed by BALB/c and then CBA.

Figure 6.14 Numbers of Null Cells in the Spleen

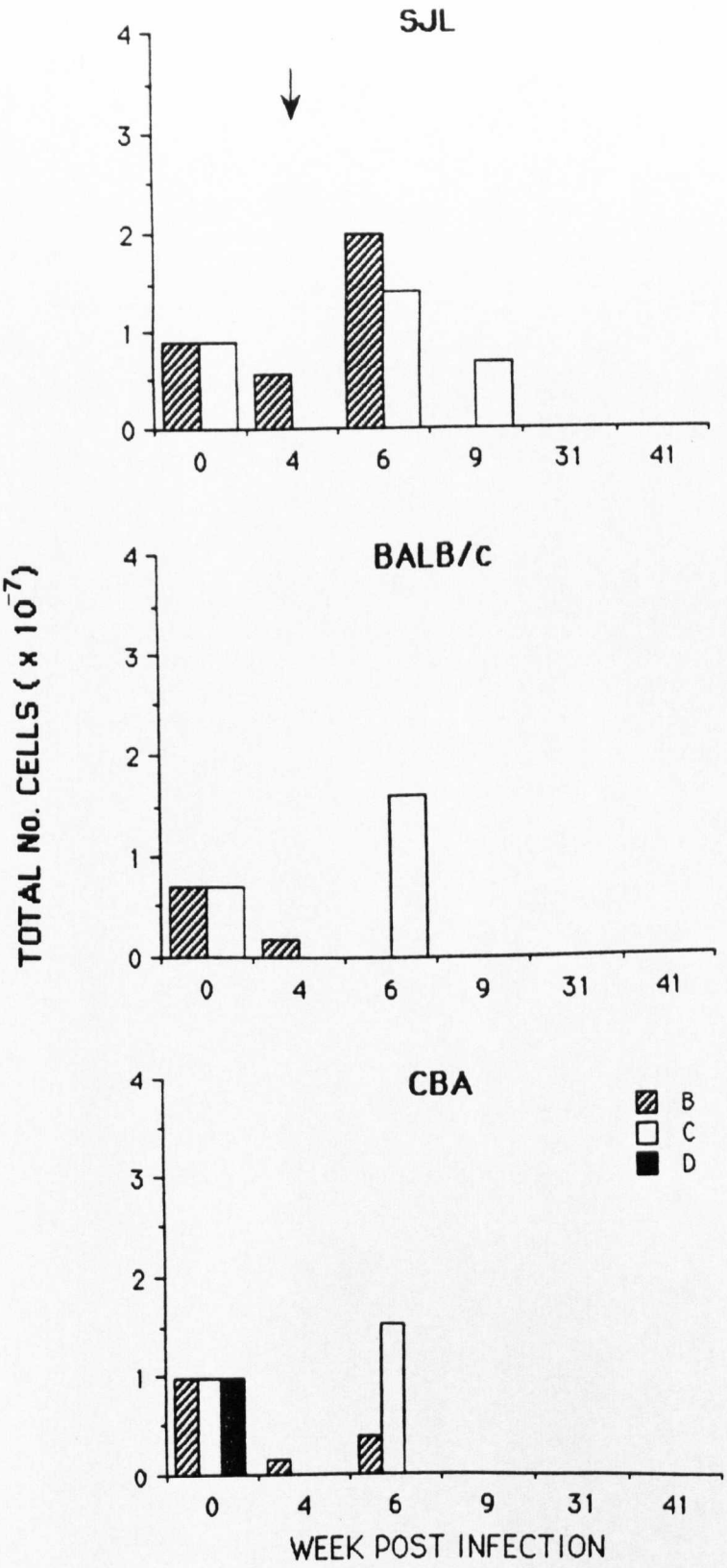
The total numbers of viable null cells present in the spleens of immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice was calculated by assessing which cells were not recognised by either B or T cell markers. The time of challenge is indicated by an arrow.


SJL
BALB/c
CBA
TOTAL No. CELLS (x 10⁻⁷)
WEEK POST INFECTION
B
C
D
0
2
4
6
8
10
0
4
6
9
31
41

The numbers of these cells in the MLN (Fig. 6.15) rose in group B of SJL until the parasite was expelled whereas the unimmunised group, C, rose post challenge and then fell when the worm was lost. Those in BALB/c B decreased to zero whereas in group C the numbers of cells doubled. In all groups of CBA the numbers of cells declined for the duration of the experiment.

Figure 6.15 Numbers of Null Cells in the Mesenteric Lymph Nodes

The total numbers of viable null cells present in the MLN of immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice was calculated by assessing which cells were not recognised by either B or T cell markers. The time of challenge is indicated by an arrow.


SJL
BALB/c
CBA
TOTAL No. CELLS (x 10^{-7})
WEEK POST INFECTION
B
C
D
0
1
2
3
4
0
4
6
9
31
41

6.2.6 Blastogenic Response of the Secondary Lymphoid Organs

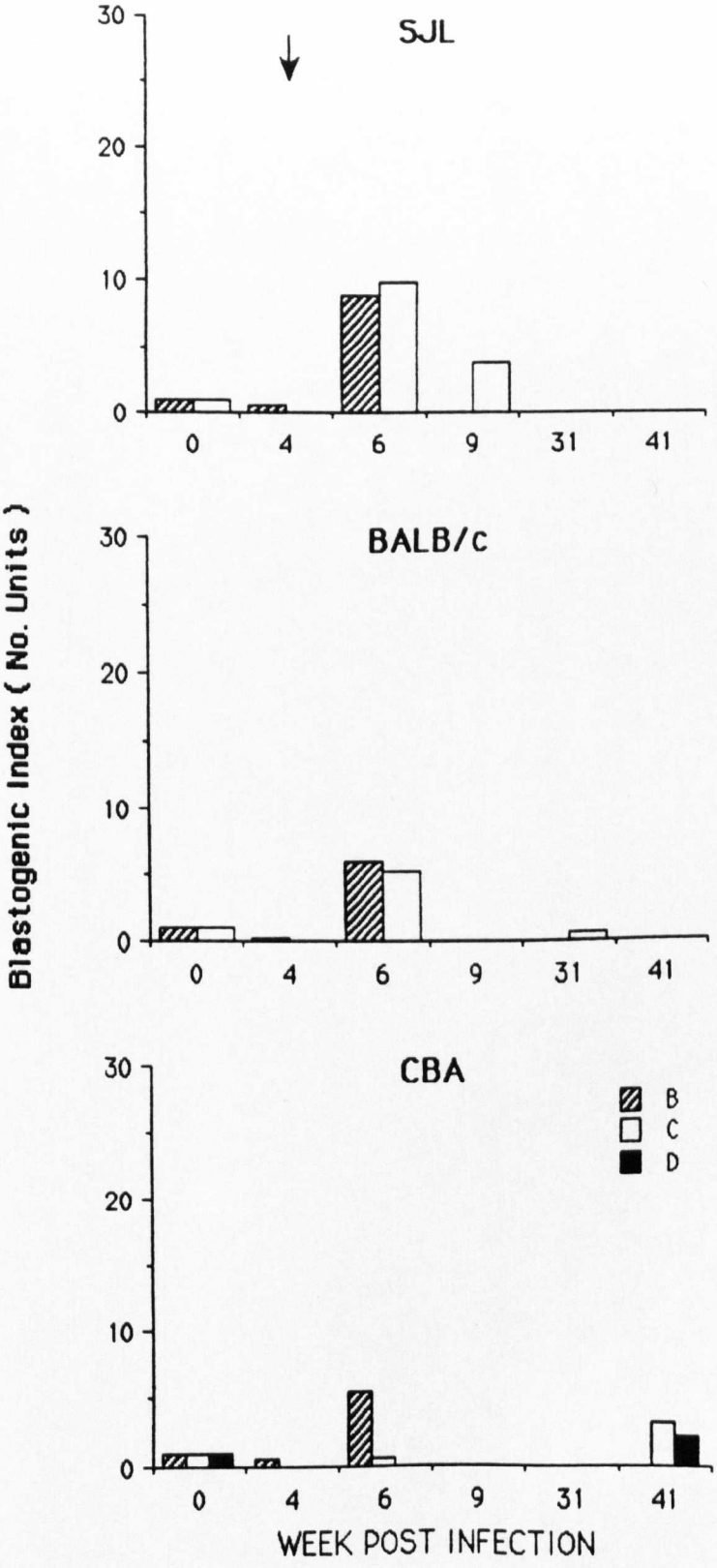
The blastogenic response of the viable cells of the spleen and the MLN was assessed as described in Chapter 5. The response of the cells from the spleen (Fig. 6.16) in group B of all the strains did not increase until after challenge, then SJL were more responsive than BALB/c which in turn, were more responsive than CBA. In each strain the response of the unimmunised group, C, rose after challenge but then fell when the worm was rejected.

Figure 6.16 Blastogenesis of the Cells of the Spleen

The blastogenesis of the viable cells from the spleen of immunised (B and D) and unimmunised (C) groups of SJL, BALB/c and CBA was assessed. Cells were taken at autopsy along with those from naive animals and cultured in medium containing 10% FCS. After labelling with tritiated thymidine the blastogenic index was calculated thus;

$$\text{Index} = \frac{\text{Thymidine incorporation of infected cells}}{\text{Thymidine incorporation of naive cells}}$$

The point of challenge is shown by the arrow.

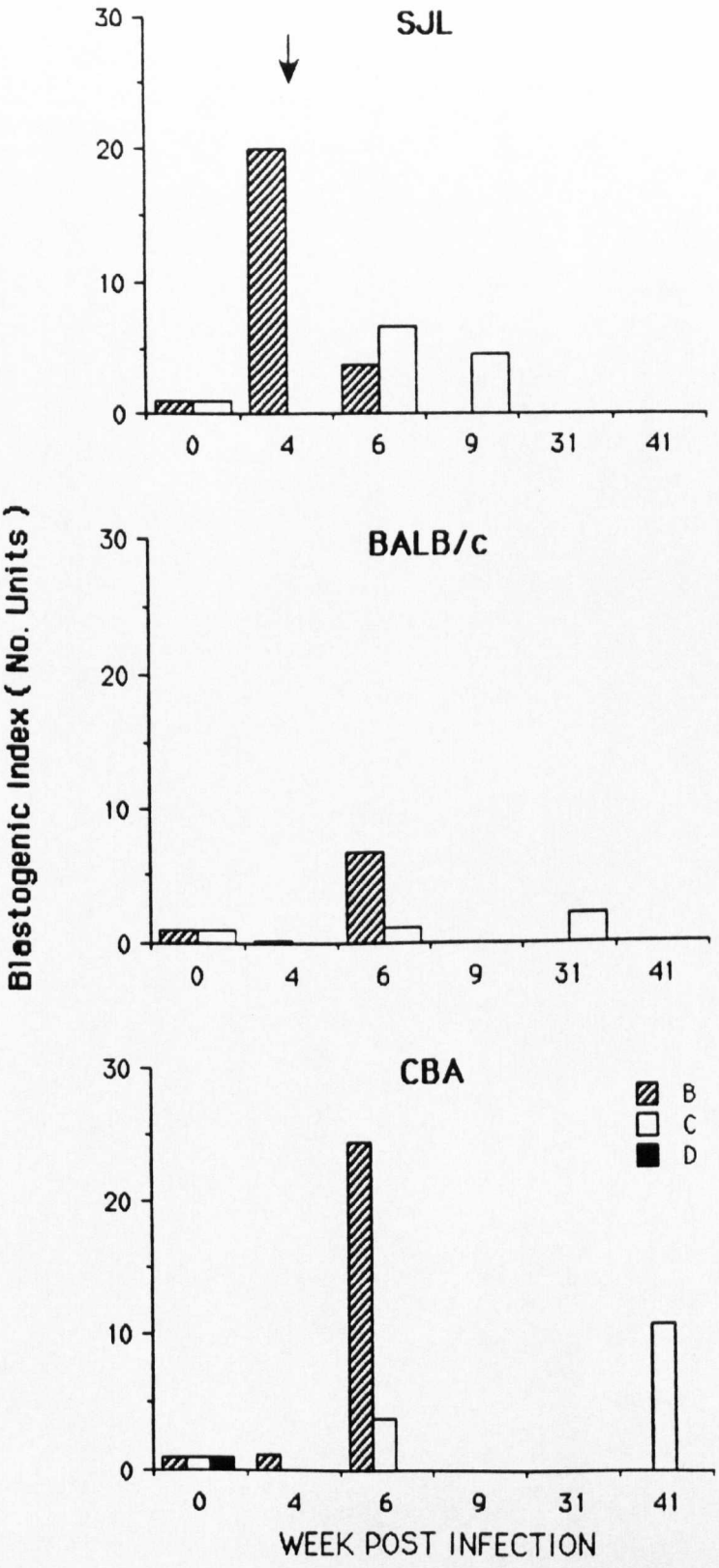

SJL
BALB/c
CBA
30
20
10
0
0
4
6
9
31
41
B
C
D
Blastogenic Index (No. Units)
WEEK POST INFECTION

The MLN cells (Fig. 6.17) showed a much higher reactivity than the spleen cells. In BALB/c and CBA the reactivity of the immunised group, B, was highest after challenge whilst that of group C was greatest when the parasite was expelled. In SJL, the response of group B was highest before challenge and that of group C was highest after challenge and had declined by the time the worm was lost.

Figure 6.17 Blastogenesis of the Cells of the Mesenteric Lymph Nodes

The blastogenesis of the viable cells from the MLN of immunised (B and D) and unimmunised (C) groups of SJL, BALB/c and CBA was assessed. Cells were taken at autopsy along with those from naive animals and cultured in medium containing 10% FCS. After labelling with tritiated thymidine the blastogenic index was calculated. The point of challenge is shown by the arrow.

$$\text{Index} = \frac{\text{Thymidine incorporation of infected cells}}{\text{Thymidine incorporation of naive cells}}$$


SJL
BALB/c
CBA
Blastogenic Index (No. Units)
30
20
10
0
0
4
6
9
31
41
B
C
D
WEEK POST INFECTION

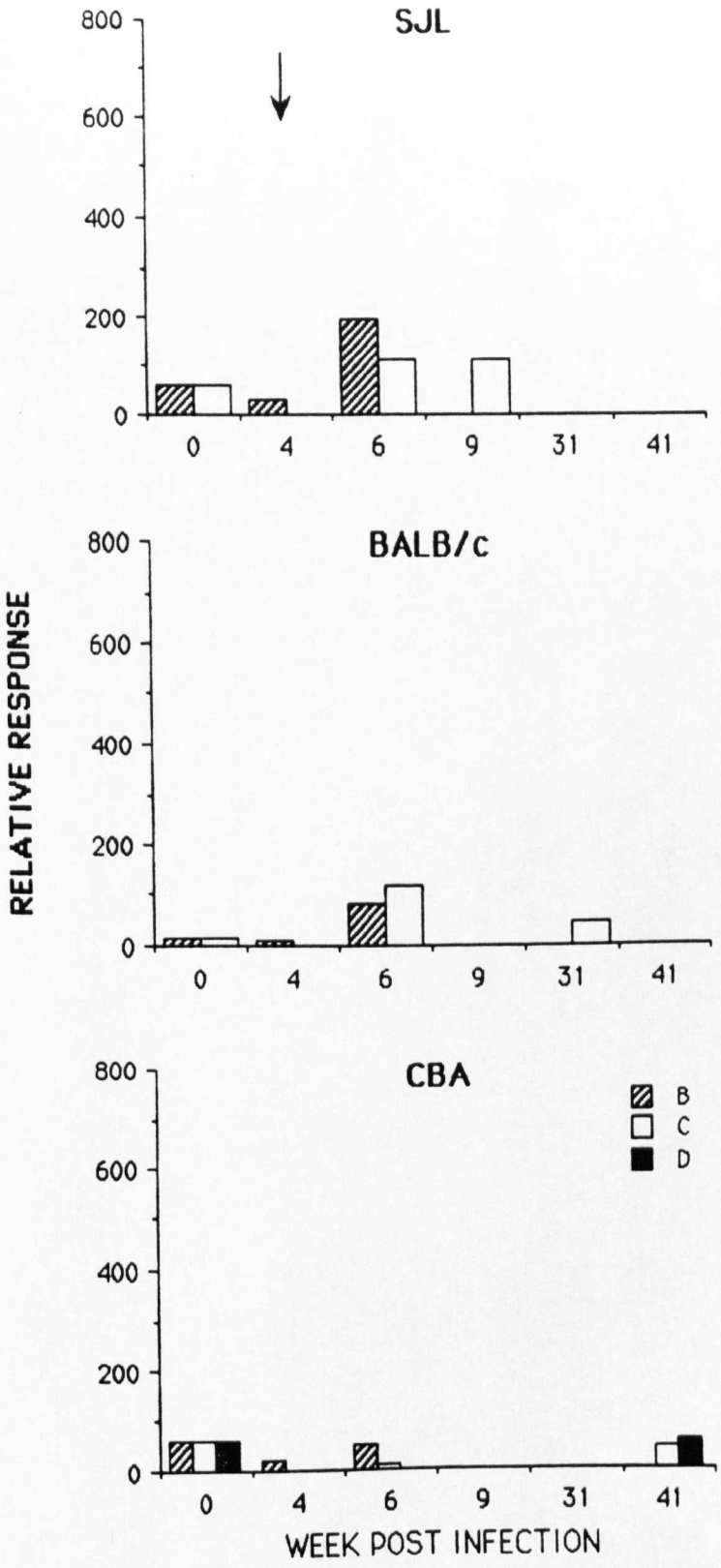
6.2.7 Response to Mitogen

The ability of the viable cells from the spleen and MLN to respond to two mitogens was also investigated. Fig. 6.18 illustrates the response of spleen cells to the T cell mitogen, Con A. It was seen that there was no change in any of the groups of CBA. Both groups of BALB/c and SJL rose after challenge but the response of the unimmunised group, C, fell as the parasite was expelled.

Figure 6.18 Response of Cells of the Spleen to the Mitogen, Con A

The response of the viable cells from the spleens of immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice to the T cell mitogen, Con A was assessed following the culture of 5 x 10^6 cells with lug of Con A for 24 hours at 37°C prior to the addition of tritiated thymidine.The response of the infected cells relative to that of the naive cells was calculated. The point of challenge is indicated by the arrow.

$$\text{Relative Response} = \frac{\text{cpm of mitogen stimulated cells}}{\text{cpm of unstimulated cells}}$$

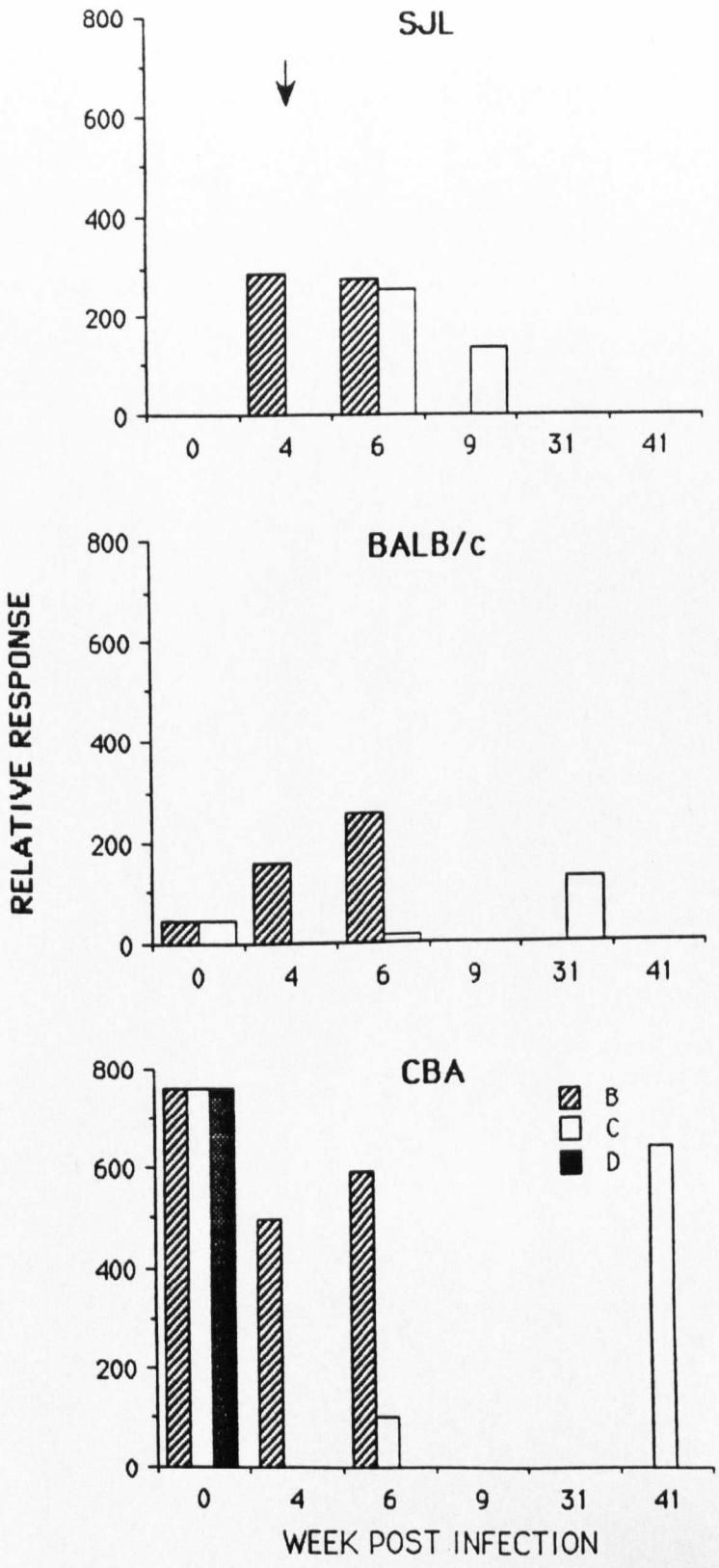

SJL
BALB/c
CBA
800
600
400
200
0
0
4
6
9
31
41
B
C
D
RELATIVE RESPONSE
WEEK POST INFECTION

Overall, the response of the cells from the MLN to the same mitogen (Fig. 6.19) was much greater than that of the spleen cells. The reaction of both groups of SJL rose after infection, but that of group C fell until the parasite was lost. The response of group B of BALB/c rose after challenge whereas that of group C did not rise until the parasite had been expelled and then not to the same degree. The response of CBA was very high throughout the experiment although there was a slight drop in the response of group B before challenge, followed by a rise but not to the initial levels. The reaction of group C dropped drastically after challenge but was restored to initial levels by the time the parasite was expelled.

Figure 6.19 Response of the Cells of the Mesenteric Lymph Nodes to the Mitogen, Con A

The response of the viable cells from the MLN of immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice to the T cell mitogen, Con A was assessed following the culture of 5×10^6 cells with lug of Con A for 24 hours at 37^oC prior to the addition of tritiated thymidine.The response of the infected cells relative to that of the naive cells was calculated. The point of challenge is indicated by the arrow.

$$\text{Relative Response} = \frac{\text{cpm of mitogen stimulated cells}}{\text{cpm of unstimulated cells}}$$


SJL
BALB/c
CBA
RELATIVE RESPONSE
800
600
400
200
0
0
4
6
9
31
41
B
C
D
WEEK POST INFECTION

Overall, the response of the cells from the spleen and the MLN to LPS, a B cell mitogen, was much lower than that to Con A (Figs. 6.20 and 6. 21). The response of SJL and BALB/c both rose after challenge although the response of group C, the unimmunised group, fell as the parasite was expelled. Group C and D of CBA both increased at the time of expulsion but that of group B was maximal before challenge. The response of the MLN cells to LPS showed the same pattern of response as for the spleen though to a lesser degree.

Figure 6.20 Response of the Cells of the Spleen to the Mitogen, LPS

The response of the viable cells from the spleens of immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice to the B cell mitogen, LPS was assessed following the culture of 5×10^6 cells with 1ug of LPS for 24 hours at 37^oC prior to the addition of tritiated thymidine.The response of the infected cells relative to that of the naive cells was calculated. The point of challenge is indicated by the arrow.

$$\text{Relative Response} = \frac{\text{cpm of mitogen stimulated cells}}{\text{cpm of unstimulated cells}}$$

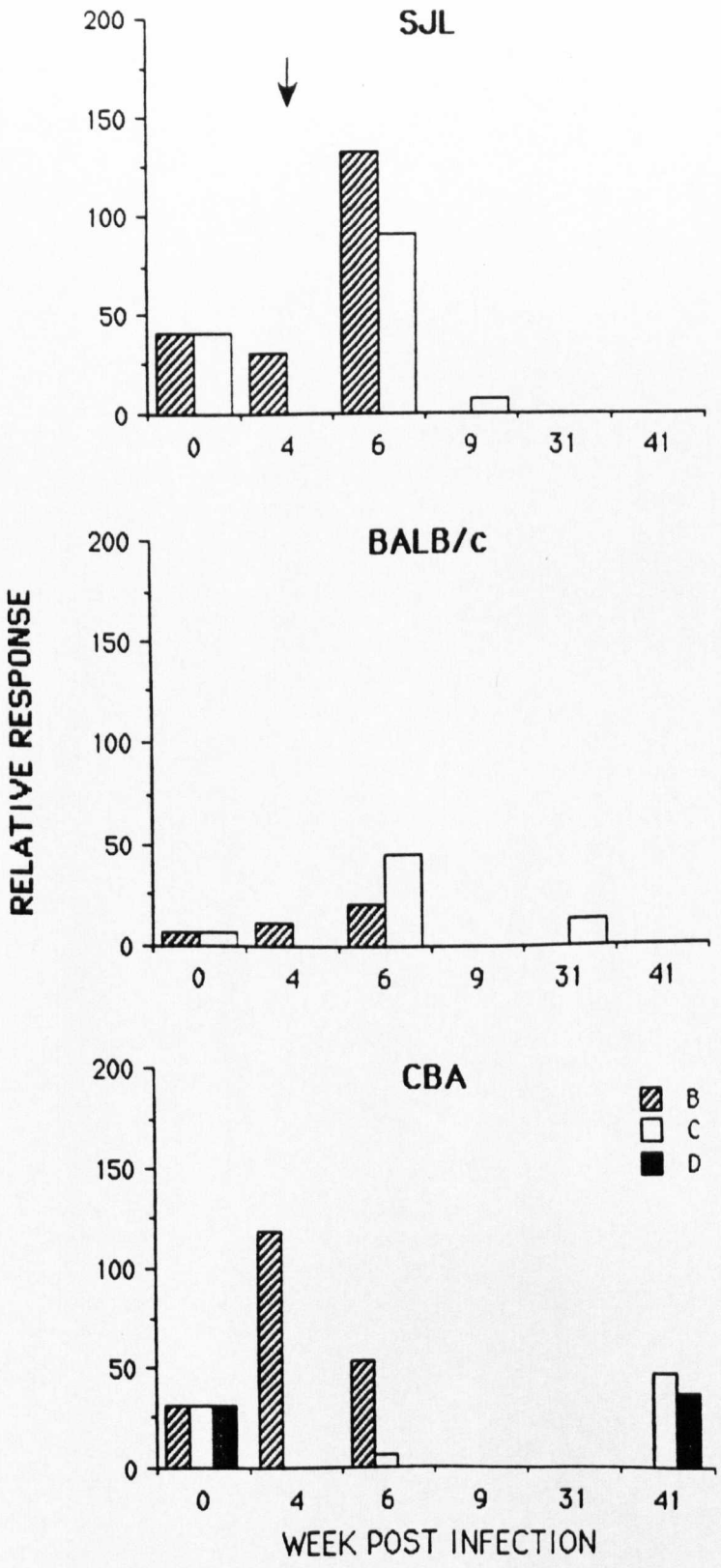
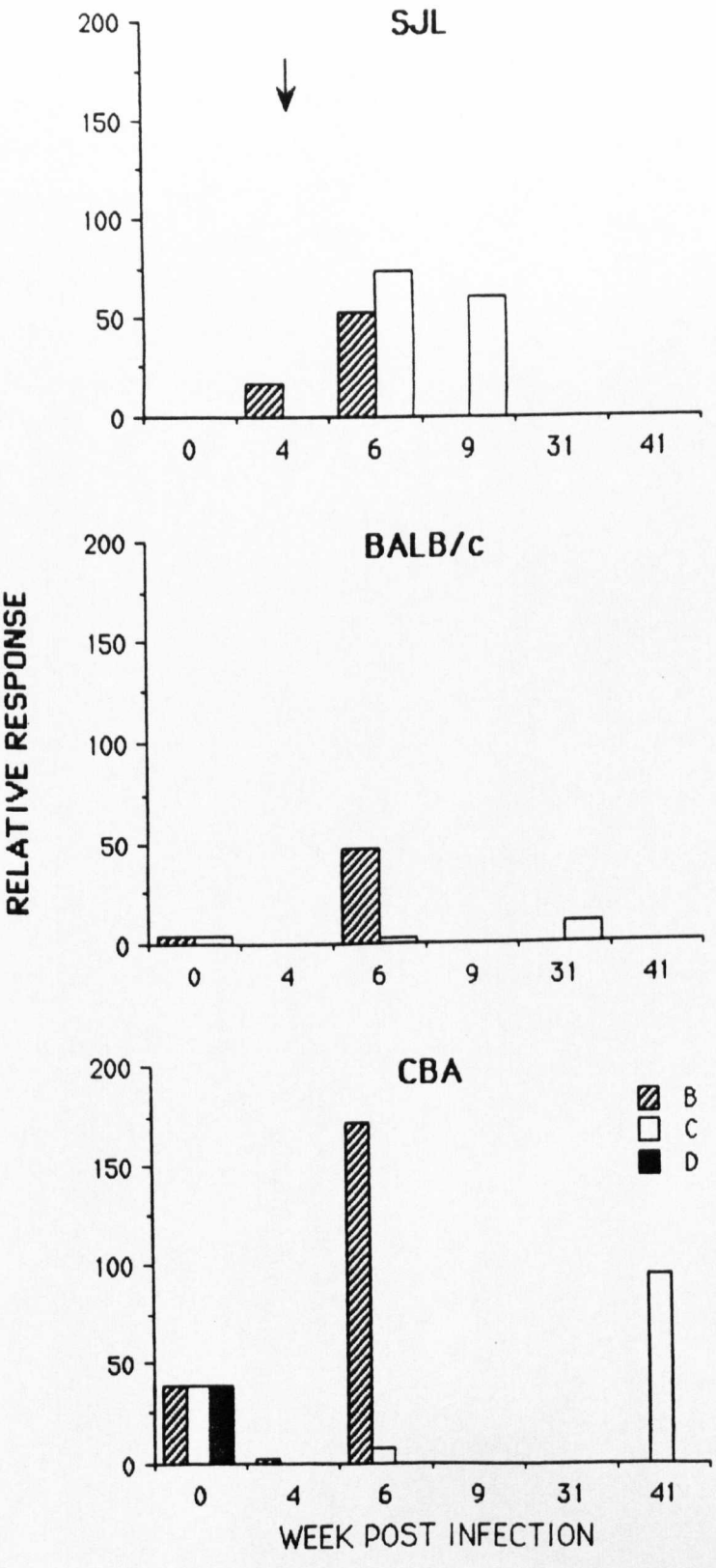

SJL
BALB/c
CBA
RELATIVE RESPONSE
200
150
100
50
0
0
4
6
9
31
41
B
C
D
WEEK POST INFECTION

Figure 6.21 Response of the Cells of the Mesenteric Lymph Node to the Mitogen, LPS

The response of the viable cells from the MLN of immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice to the B cell mitogen, LPS was assessed following the culture of 5×10^6 cells with lug of LPS for 24 hours at $37^{o}C$ prior to the addition of tritiated thymidine.The response of the infected cells relative to that of the naive cells was calculated. The point of challenge is indicated by the arrow.

$$\text{Relative Response} = \frac{\text{cpm of mitogen stimulated cells}}{\text{cpm of unstimulated cells}}$$


SJL
BALB/c
CBA
RELATIVE RESPONSE
200
150
100
50
0
0
4
6
9
31
41
B
C
D
WEEK POST INFECTION

6.2.8 Mucosal Mast Cell Response

The response of the effector cells in the gut mucosa to infection was examined by enumerating the mucosal mast cell response (Fig. 6.22). In each strain there was little or no mastocytosis in the immunised group, B. However, in group C, it was noted that SJL experienced a gradual increase in the numbers of mast cells until at the time of worm expulsion it was eight times that of the control level. Group C of BALB/c, showed a slight increase, double that of the control levels, after challenge, but this had declined at the time of worm expulsion. CBA did not display any mastocytosis at any point in the infection.

Figure 6.22 Numbers of Mast Cells Present in the Gut

The numbers of mucosal mast cells present in the mucosa of immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice was measured by staining gut sections obtained at each autopsy with Alcian/Astra Blue stain and counterstaining with Safranin O. The numbers of cells present in 25 villus crypt units (VCU) was counted per mouse and the mean and standard error of the number of cells present per VCU was calculated for each group. The point of challenge is indicated by an arrow.

SJL

B0 vs B6: $p < 0.05$ C0 vs C6: $p < 0.05$

C0 vs C9: $p < 0.05$ C6 vs C9: NS

C6 vs B6: $p < 0.05$

BALB/c

B0 vs B6: $p < 0.05$ C0 vs C6: NS

C0 vs C31: $p < 0.005$ C6 vs C31: NS

B6 vs C6: NS

CBA

B0 vs B6: $p < 0.05$ C0 vs C6: $p < 0.05$

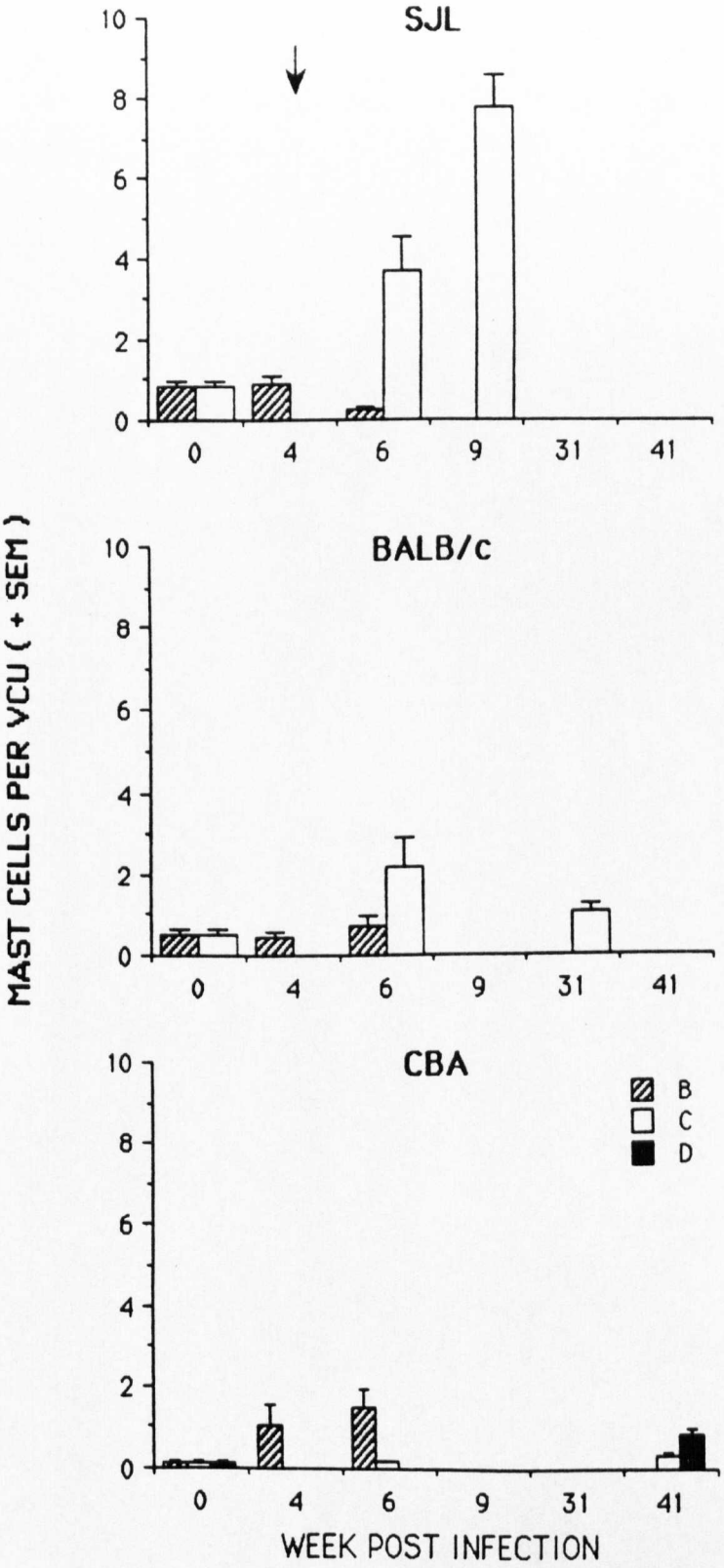
C0 vs C41: $p < 0.005$ C6 vs C41: $p < 0.005$

SJL C6 vs BALB/c C6: NS SJL C6 vs CBA C6: NS

SJL B6 vs CBA B6: $p < 0.05$ SJL B6 vs BALB/c B6: NS

SJL C9 vs CBA C41: NS

N.B. NS (
not significant)


SJL
BALB/c
CBA
B
C
D
MAST CELLS PER VCU (+ SEM)
WEEK POST INFECTION
0
2
4
6
8
10
0
4
6
9
31
41

6.2.9 Antibody Response to Parasite Antigens

The humoral response of the host to the parasite was also examined using an ELISA as for Chapter 5. The response to adult ES antigens is shown (Fig. 6.23). The response of both groups of SJL rose considerably after the challenge infection, more so in group C. However, this response had declined by the time the parasite was expelled. In BALB/c, the response of both groups was very similar, but that of group C, the unimmunised group, continued to increase until the worm was rejected. With CBA the response was very similar to that of BALB/c with the same response being attained at the time of parasite loss, but taking longer to reach these levels.

Figure 6.23 Antibody Response To Adult ES Products

The quantitative antibody response of sera from immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice to adult ES products was measured using an ELISA with a serum concentration of 1:100 and an antigen concentration of 0.25 ug/well. The reaction was developed using alkaline phosphatase anti-mouse IgGAM. The mean and the standard error for each time point was calculated. The point of challenge is represented by an arrow.

SJL

B0 vs B6: $p < 0.05$ C0 vs C6: $p < 0.05$

C0 vs C9: $p < 0.005$ B6 vs C6: $p < 0.05$

BALB/c

B0 vs B6: $p < 0.05$ C0 vs C6: $p < 0.05$

C0 vs C31: $p < 0.05$ B6 vs C6: NS

CBA

B0 vs B6: $p < 0.005$ C0 vs C6: $p < 0.005$

C0 vs C41: $p < 0.05$ B6 vs C6: NS

D0 vs D41: p , 0.05 C41 vs D41: NS

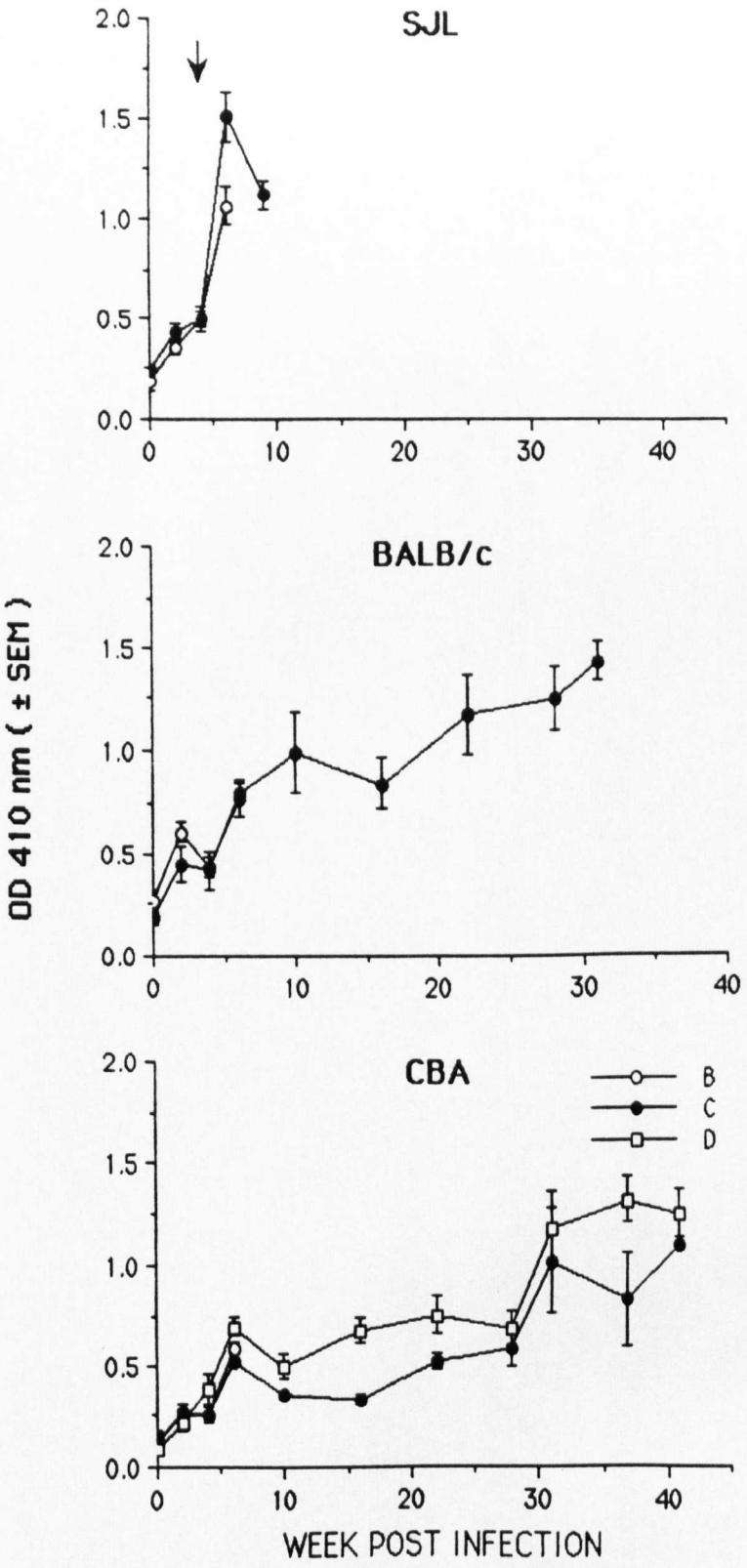
SJL B6 vs BALB/c B6: NS SJL C6 vs BALB/c C6: $p < 0.05$

CBA B6 vs BALB/c B6: NS CBA C6 vs BALB/c C6: $p < 0.05$

SJL B6 vs CBA B6: $p < 0.05$ SJL C6 vs BALB/c C31: NS

SJL C6 vs CBA C41: $p < 0.05$

N.B. NS (not significant)


SJL
BALB/c
CBA
B
C
D
OD 410 nm (± SEM)
WEEK POST INFECTION
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40

When the response to larval ES was analysed (Fig. 6.24) it can be seen that the reaction of all of the groups appeared to mirror the response to adult antigens.

Figure 6.24 Antibody Response to Larval ES Products

The quantitative antibody response of sera from immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice to day 4 larval ES products was measured using an ELISA with a serum concentration of 1:100 and an antigen concentration of 0.25 ug/well. The reaction was developed using alkaline phosphatase anti-mouse IgGAM. The mean and the standard error for each time point was calculated. The point of challenge in represented by an arrow.

SJL

B0 vs B6: $p < 0.05$	C0 vs C6: $p < 0.05$
C0 vs C9: $p < 0.005$	B6 vs C6: NS

BALB/c

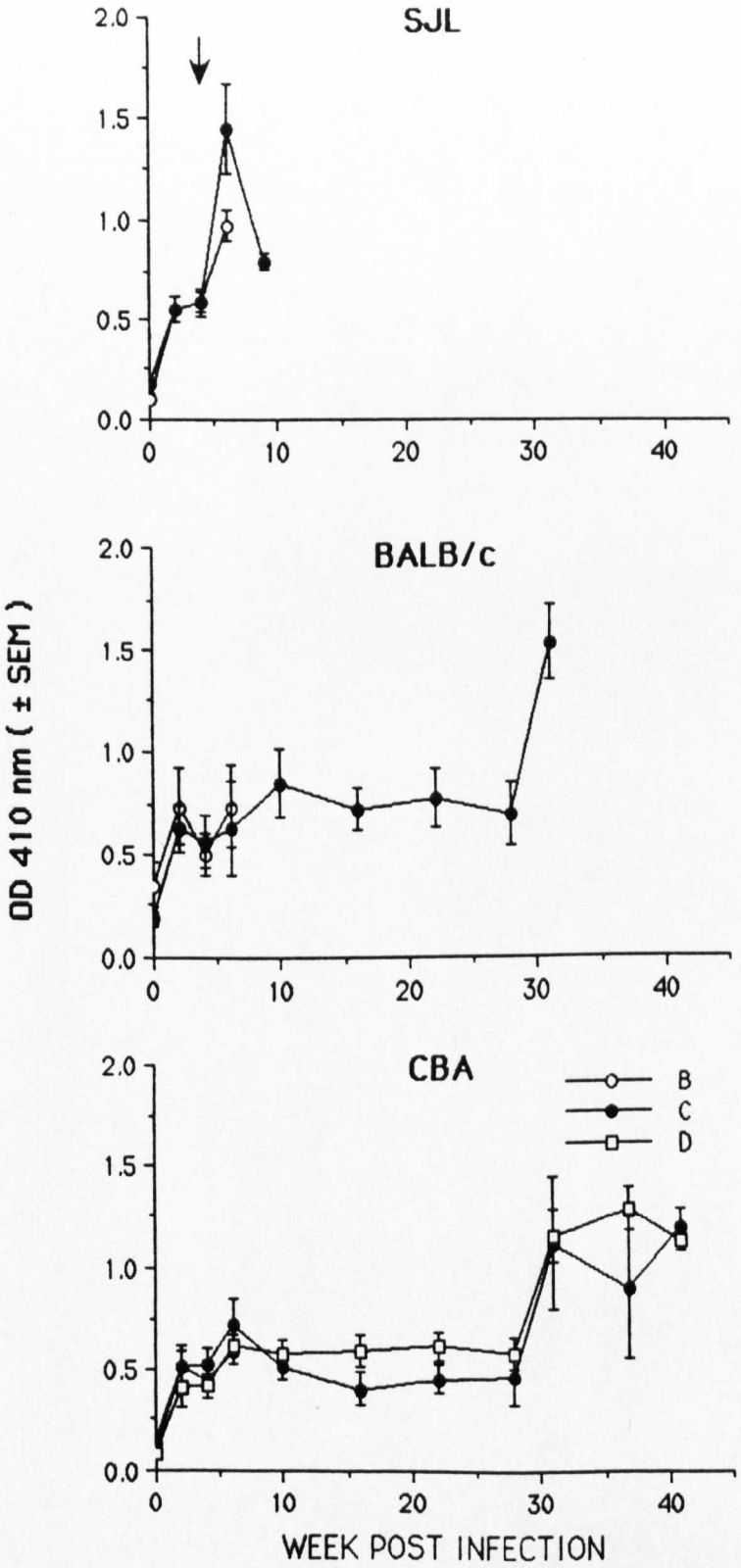
B0 vs B6: NS	C0 vs C6: NS
C0 vs C31: $p < 0.05$	B6 vs C6: NS

CBA

B0 vs B6: $p < 0.05$	C0 vs C6: $p < 0.05$
C0 vs C41: NS	B6 vs C6: NS
D0 vs D41: $p < 0.05$	C41 vs D41: NS

SJL B6 vs BALB/c B6: NS	SJL C6 vs BALB/c C6: NS
SJL B6 vs CBA B6: $p < 0.05$	SJL C6 vs CBA C6: NS

N.B. NS (not significant)

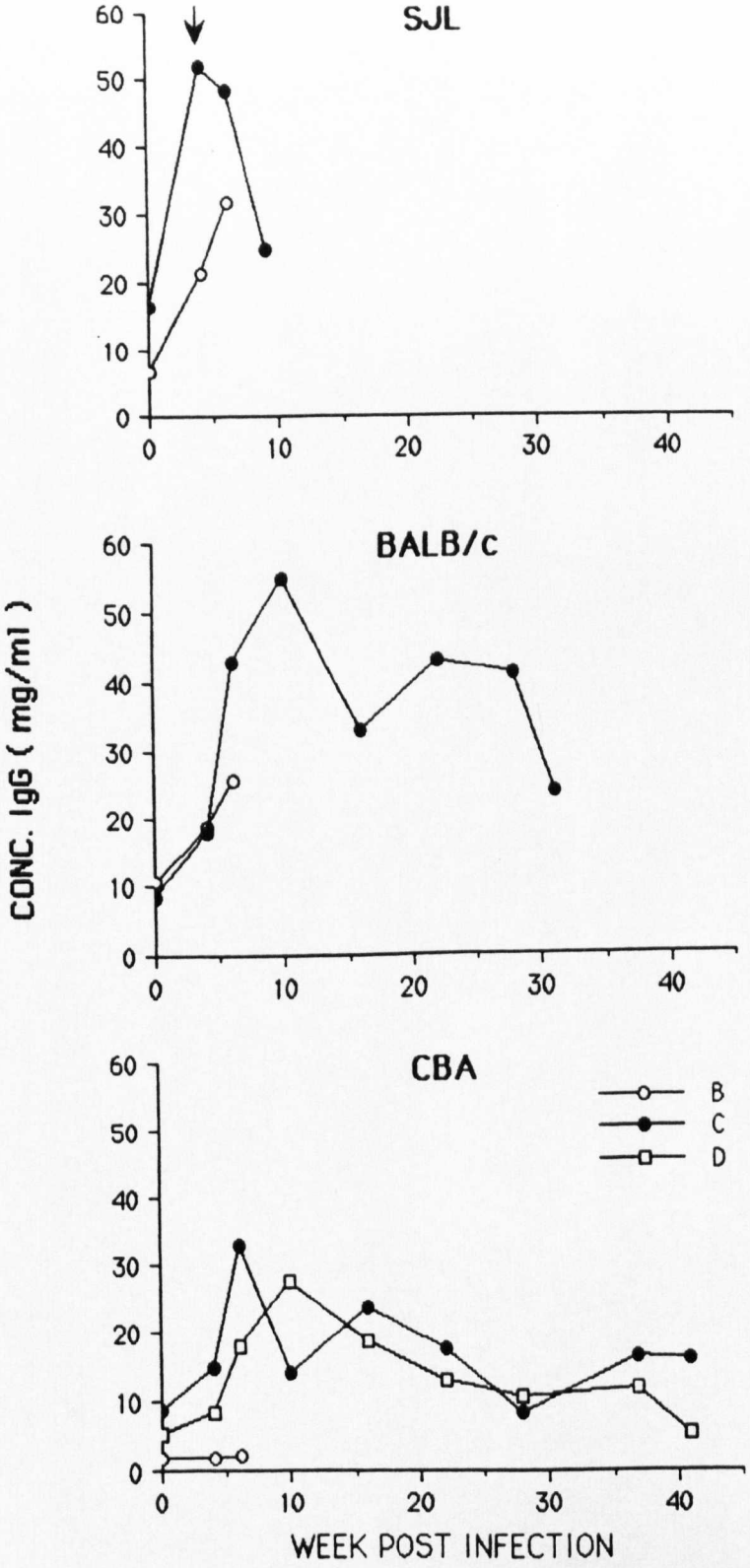

SJL
BALB/c
CBA
B
C
D
OD 410 nm (± SEM)
WEEK POST INFECTION
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40

6.2.10 Concentration of Antibody Classes

In order to analyse which antibody classes were involved in the response against the parasite, the concentrations of IgG, IgA and IgM were calculated using the Mancini technique. The concentration of IgG (Fig. 6.25) in SJL mirrored the antibody response to parasite antigens in an ELISA, however, that of BALB/c and CBA did not. Groups C and D of BALB/c and CBA rose sharply after challenge but then declined until the parasite was lost. The response of group B did not appear to change in CBA and only rose slightly in BALB/c.

Figure 6.25 IgG Concentration

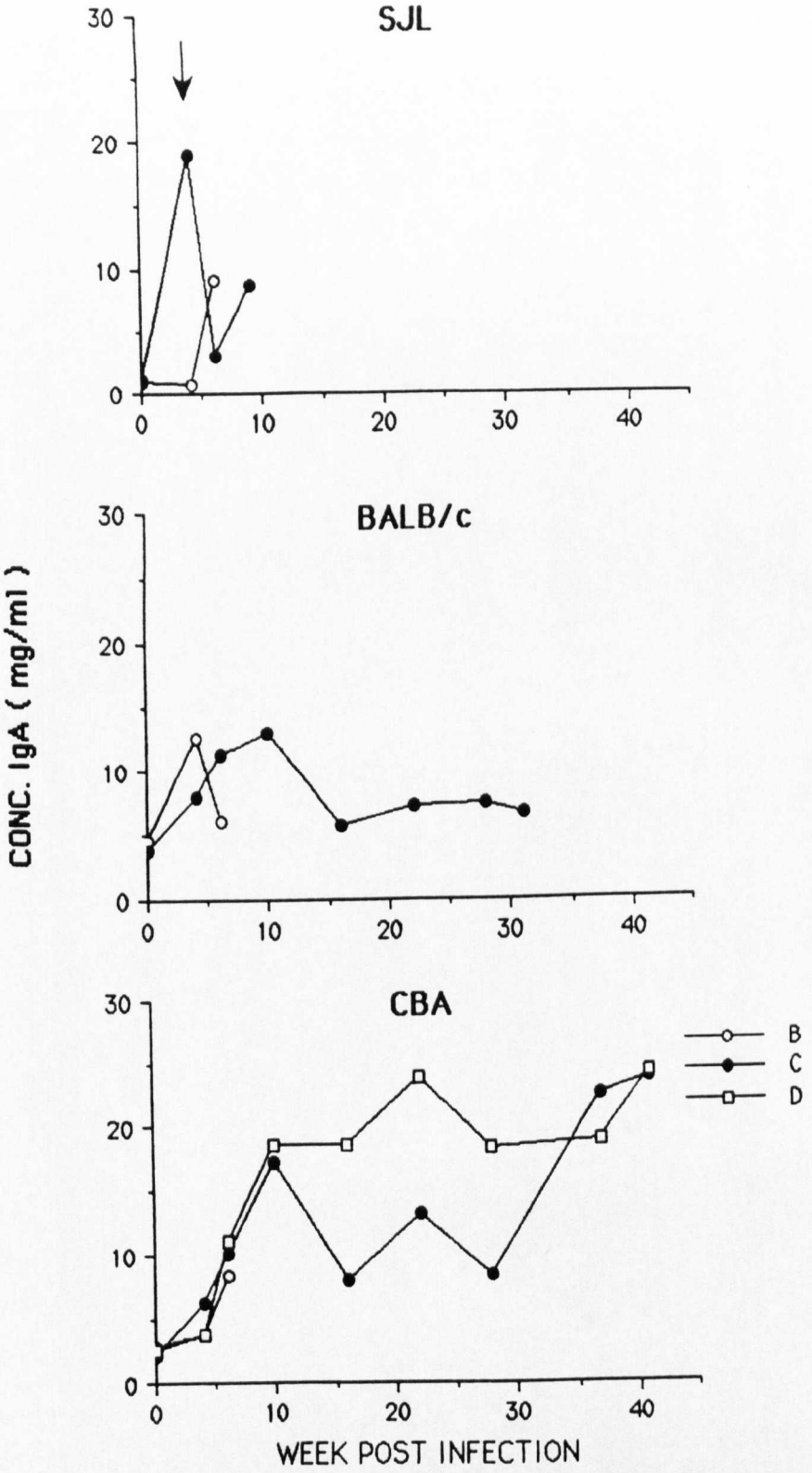
The concentration of the antibody IgG was measured in the sera of immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice at various time points through the experiment using a single radial immunodiffusion kit (The Binding Site Ltd.). The concentration was calculated and expressed as mg immunoglobulin per ml serum. The point of challenge is indicated by the arrow.


SJL
BALB/c
CBA
B
C
D
CONC. IgG (mg/ml)
WEEK POST INFECTION
0
10
20
30
40
50
60

The IgA response (Fig. 6.26) was much lower than that of IgG and displayed a different pattern of response. In each case, there was a rise in the response of the immunised group, B, after challenge. In group C of SJL and BALB/c the levels rose after challenge and then fell to normal levels by the time the parasite was lost. The response of CBA groups C and D rose throughout the infection mirroring the response to parasite antigens on ELISA.

Figure 6.26 IgA Concentration

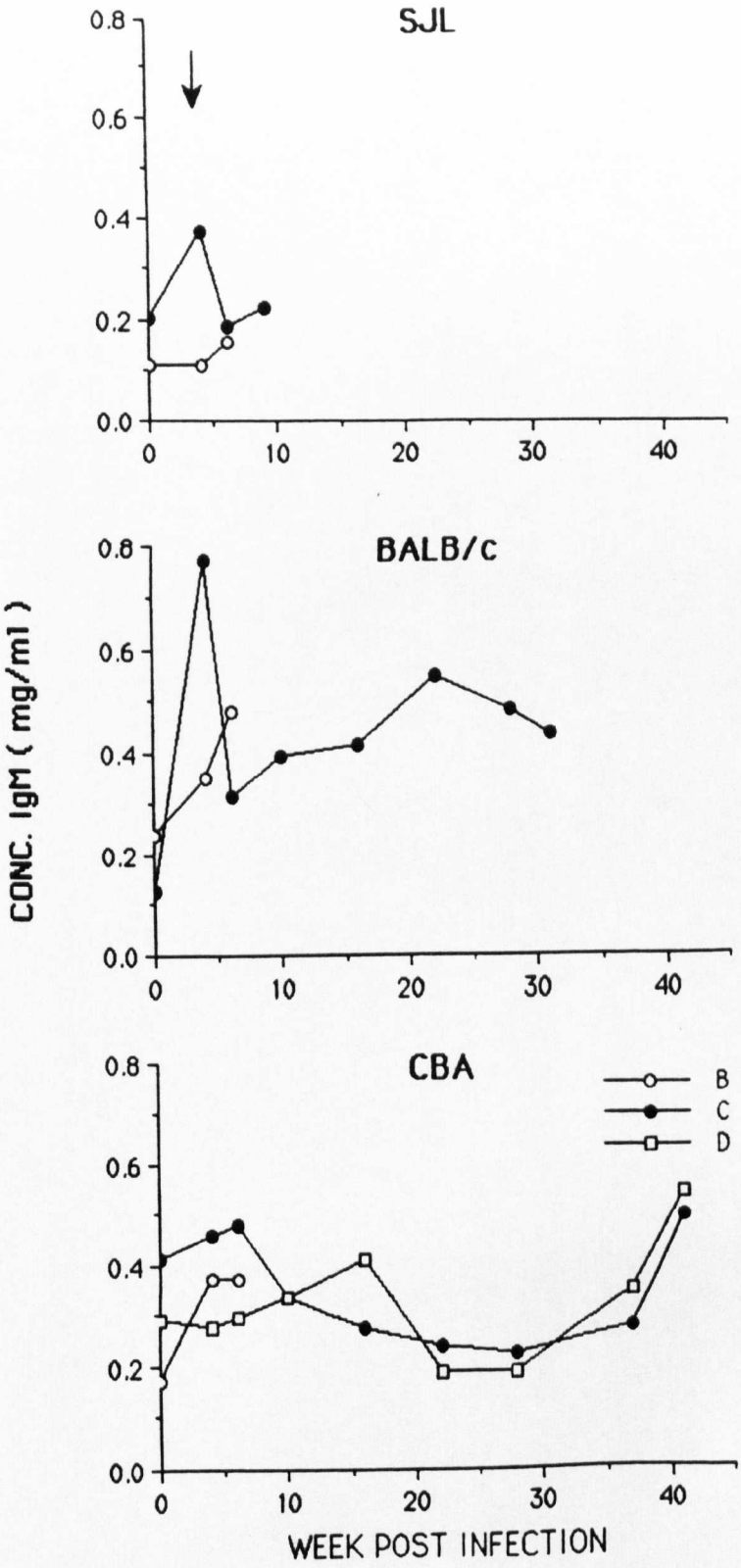
The concentration of the antibody IgA was measured in the sera of immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice at various time points through the experiment using a single radial immunodiffusion kit (The Binding Site Ltd.). The concentration was calculated and expressed as mg immunoglobulin per ml serum. The point of challenge is indicated by the arrow.


SJL
BALB/c
CBA
CONC. IgA (mg/ml)
WEEK POST INFECTION
B
C
D
0
10
20
30
40

The concentrations of IgM (Fig. 6.27) was minimal, however, the pattern of response was very similar to that seen in the IgA response. The response of the immunised groups, B, all rose after challenge as did that of group C of SJL and BALB/c, but these had declined to normal levels by the time the parasite was expelled. The response of CBA, although not extensive, appeared to continue increasing until the parasite was lost.

Figure 6.27 IgM Concentration

The concentration of the antibody IgM was measured in the sera of immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice at various time points through the experiment using a single radial immunodiffusion kit (The Binding Site Ltd.). The concentration was calculated and expressed as mg immunoglobulin per ml serum. The point of challenge is indicated by the arrow.


SJL
BALB/c
CBA
B
C
D
CONC. IgM (mg/ml)
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
WEEK POST INFECTION

6.2.11 Qualitative Antibody Response to Parasite Antigens

Sera from various time points in the experiment was used to probe Western blots of adult ES and homogenate and larval ES. From this it can be seen that very few molecules were recognised in either adult ES or homogenate (Figs. 6.28 and 6.29), in contrast to the strong response noted in the primary infection (Chapter 5). The molecules that were recognised were of weights 67 and 30.5 kDa in the homogenate and 67 and 13 kDa in the ES. When the larval preparation was probed no molecules at all were recognised.

Figure 6.28 Immunoblot of Adult Homogenate Probed with Immunised or Unimmunised Mouse Sera

1mg of day 10 adult homogenate was run under reducing conditions on a 5-20% gel. Following electrophoretic transfer to a sheet of nitrocellulose, the resulting immunoblot was cut into 5mm strips and probed with sera at a dilution of 1:250 from immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice and developed with HRP-sheep anti-mouse IgGAM. Values for molecular weight standards are shown on the left hand side.

Lane 1	Naive SJL	
" 2	Immunised SJL (B)	week 6 p.i.
" 3	Unimmunised SJL (C)	week 9 p.i.
" 4	Naive BALB/c	
" 5	Immunised BALB/c (B)	week 6 p.i.
" 6	Unimmunised BALB/c (C)	week 31 p.i.
" 7	Naive CBA	
" 8	Immunised CBA (B)	week 6 p.i.
" 9	Immunised CBA (D)	week 41 p.i.
" 10	Unimmunised CBA (C)	week 41 p.i.

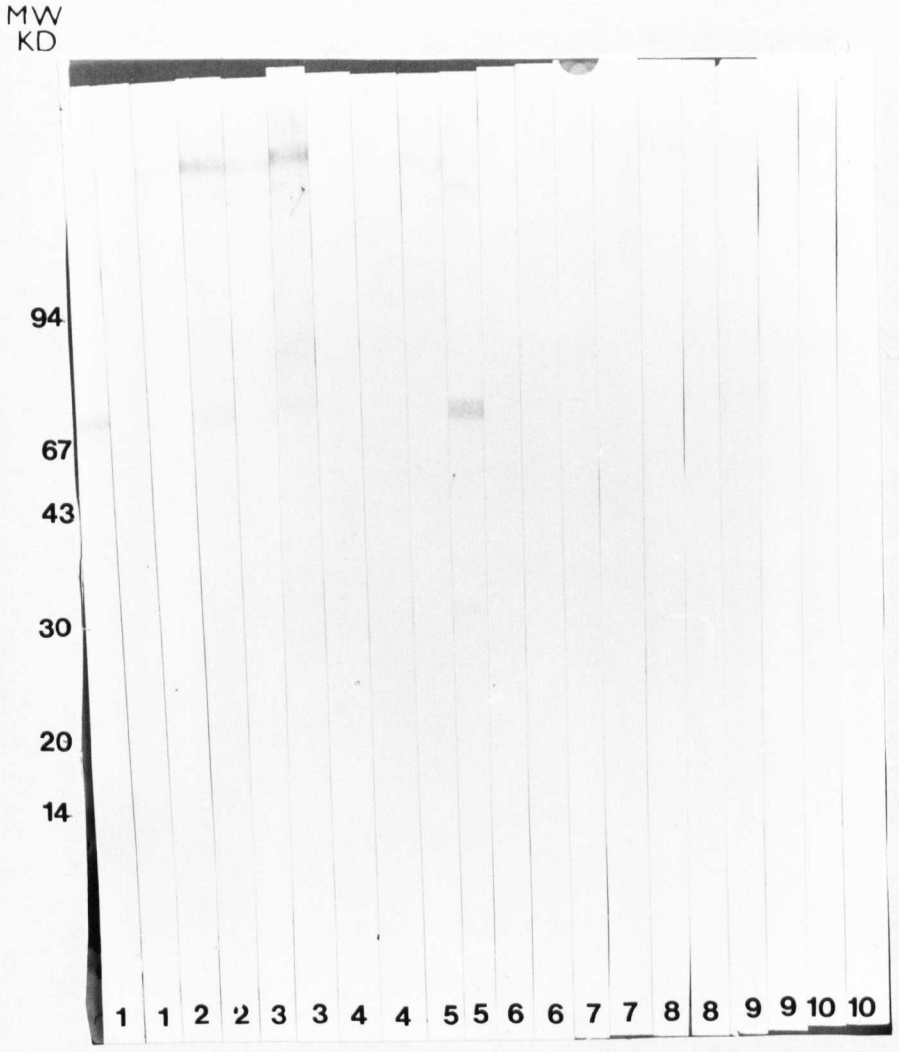
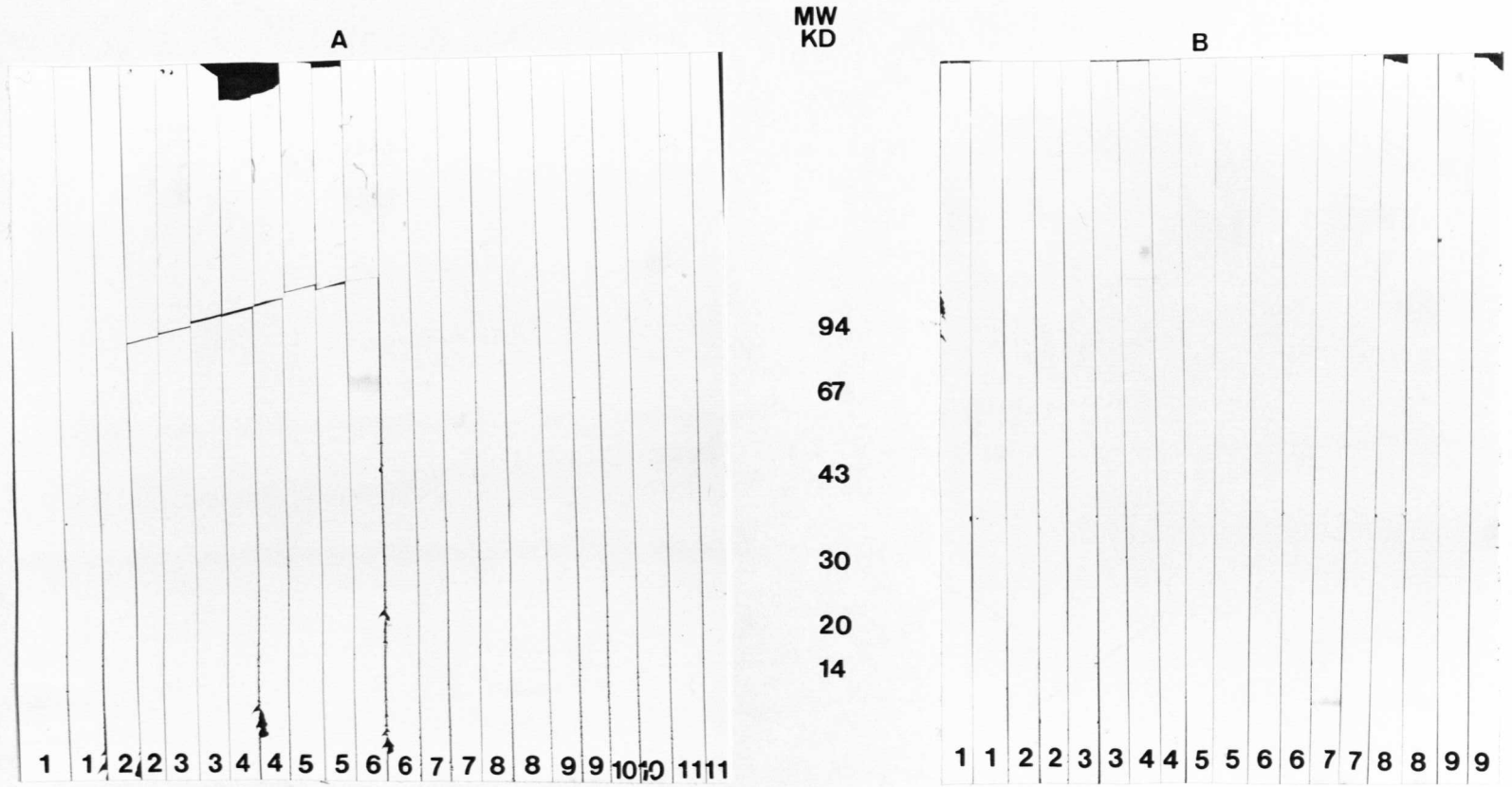

MW
KD
94
67
43
30
20
14
1 1 2 2 3 3 4 4 5 5 6 6 7 7 8 8 9 9 10 10

Figure 6.29 Immunoblot of Adult ES Products Probed with Immunised or Unimmunised Mouse Sera

1mg of day 10 adult ES products was run under reducing conditions on a 5-20% gel. Following electrophoretic transfer to a sheet of nitrocellulose, the resulting immunoblot was cut into 5mm strips and probed with sera at a dilution of 1:250 from immunised (B and D) and unimmunised (C) SJL, BALB/c and CBA mice and developed with HRP-sheep anti-mouse IgGAM. Values for molecular weight standards are shown on the left hand side.

(A)	Lane 1	Naive SJL	
	" 2	Immunised SJL (B)	week 4 p.i.
	" 3	" " "	week 6 p.i.
	" 4	Unimmunised SJL (C)	week 4 p.i.
	" 5	" " "	week 9 p.i.
	" 6	Naive BALB/c	
	" 7	Immunised BALB/c (B)	week 4 p.i.
	" 8	" " "	week 6 p.i.
	" 9	Unimmunised BALB/c (C)	week 4 p.i.
	" 10	" " "	week 22 p.i.
	" 11	" " "	week 31 p.i.

(B)	Lane 1	Naive CBA	
	" 2	Immunised CBA (B)	week 4 p.i.
	" 3	" " "	week 6 p.i.
	" 4	Unimmunised CBA (C)	week 4 p.i.
	" 5	" " "	week 22 p.i.
	" 6	" " "	week 41 p.i.
	" 7	Immunised CBA (D)	week 4 p.i.
	" 8	" " "	week 22 p.i.
	" 9	" " "	week 41 p.i.


A
MW
KD
94
67
43
30
20
14
1 1 2 2 3 3 4 4 5 5 6 6 7 7 8 8 9 9 10 10 11 11
B
1 1 2 2 3 3 4 4 5 5 6 6 7 7 8 8 9 9

6.3 DISCUSSION

6.3.1 Faecal Egg Counts

From the faecal egg count data it was noted that a response to the parasite was apparent from an early stage in the infection, as in the immunised group B no parasite eggs were produced before or after challenge. This suggested that either the challenge infection was not establishing, those that did were being delayed in the maturation or that the adult parasites that had established were sterile. The egg counts also indicated that the drug treatment had removed all of the parasites before they became mature adults. From group C, it was seen that the initial egg output of SJL was approximately twice that of a primary infection (see Chapter 5), but declined to zero, suggesting that any adult worms that did manage to mature did not have a reduced fecundity. Both BALB/c and CBA showed a reduced fecundity with respect to a primary infection, with the faecal egg output being about a quarter for BALB/c and a third for CBA. It was speculated that any immune reactions occurring when the host was challenged affected the fecundity of the existing population of parasites as well as the incoming challenge infection. It is possible that either expulsion of the existing adult population occurred or that their capacity to produce eggs was affected or a combination of these two factors (Enriquez, Scarpino, Cypess and Wassom, 1988). The rise in egg counts after challenge may be the result of the challenge infection establishing and starting to produce eggs or that the immunological constraints on the existing adult population had declined thus allowing the resumption of egg production by this parasite population. The fecundity of CBA group D, the immunised group, was approximately one tenth that of a primary infection for the duration of the experiments suggesting that immunity may have been acting in this group, and although the parasites may not have been rejected or prevented from establishing, the response had caused a decrease in the fecundity of the established parasites.

6.3.2 Worm Burdens

A challenge infection in SJL, group C survived for about 5 weeks which was slightly shorter (by about 1-2 weeks) than the length of time a challenge control survived in this strain (see Chapter 5) and so this would suggest that immune responses against the parasite in this strain of the mouse were acting faster than they would have done in a primary infection. In BALB/c the challenge infection lasted for about 27 weeks which was approximately double the survival time of a challenge control of a similar dose. In CBA, the infection persisted for 36 weeks which was the same length of time which a challenge control survived for in this strain of the host. The reason for the increased survival time in BALB/c could have been the result of density dependent factors acting. It has been shown previously (Robinson, Wahid, Behnke and Gilbert, 1989) that female BALB/c mice subjected to a high infection intensity with *H. polygyrus* produced a chronic infection whereas if a low intensity infection was used the parasite was expelled between 10 and 15 weeks. In this study the mice were initially infected with 200 larvae and then challenged with 100 L3 4 weeks later, thus, if all of the larvae had established from both infections, the resulting infection intensity would have been 300 worms. This number of parasites is quite sufficient to induce a chronic infection in this strain of the mouse host. An alternative explanation could be that those worms which did survive the initial host immune response were more robust, 'fitter' parasites and thus could be producing more immunomodulatory factor, or were less susceptible to the host immune response. Thus, this would either lead to hosts which were more immunodepressed or parasites which were more able to survive in the face of an immune response. Either way, this resulted in a chronic infection rather than one which was removed from the host. The longevity of the infection in CBA mice was not affected by a previous infection suggesting that no immune response had been initiated against the parasite and so it appears that loss of the parasite is more likely to be

due to natural worm senescence rather than as the result of any host immune response.

Examination of the worm burdens showed that they were initially infected with approximately three-quarters that of a comparable primary infection (see Chapter 5). This could have been the result of infection dose inaccuracies, low infectivity of the larvae due to their age or storage conditions or more resistant mice due to increased age. The most likely of these factors is a combination of the first two components as the mice used were the same age as those used for the primary infection. All the adult worms were removed from the intestine with the dose of pyrantel used thus confirming the results of Behnke and Wakelin (1977). In group C it was obvious that the challenge infection was not superimposed on to the existing parasite population and thus the parasites lost must have been from either or both of the infections, however, those worms which had established or were remaining, were lost from the host quite slowly and were not rapidly expelled. There are three possibilities for the loss of the parasites from a primary infection following a superimposed challenge infection, firstly, some of the original population may have been lost and none of the challenge infection established, secondly, all of the original population were expelled and there was a poor establishment of the challenge infection and lastly, there may have been a combination of these two factors which resulted in a decreased parasite population. In order to determine which of these hypotheses is most likely, the faeces could be monitored daily for expelled worms. Alternatively, the challenge infection could be radio-labelled. At autopsy, the proportion of the radio-labelled parasites remaining could be calculated and so the origin of the final parasite population could be determined.

6.3.3 White Blood Cell Counts

In all cases, the WBC counts rose substantially after challenge and then declined to more or less normal levels. This implied that an immune response may have acted in the early stages of the challenge infection, but declined once the stimulating larval stages had either been killed or matured to adult parasites.

6.3.4 Differential Cell Counts

Further analysis of the peripheral cellular response revealed that little or no eosinophilia occurred in any of the strains or experimental groups. This is puzzling as it has been reported by a number of authors that although an eosinophilia may not be present in a primary infection it was apparent in an immunising infection (Prowse, Ey and Jenkin, 1978). A possible explanation for the low numbers observed in this study could be that the method used to stain the cells was not as sensitive as others and so did not reveal all the cells. Because it was the peripheral eosinophilia which was assessed, it does not reveal what events are occurring in the gut; it has been shown (Liu, 1969b) that there is an intense eosinophilia and neutrophilia associated with the granuloma surrounding trapped larvae in the mucosa of the gut of immunised mice although there was no apparent peripheral eosinophilia. Thus, the parasite could be quite severely damaged by this cellular response despite it being undetected. Alternatively, it may be that the immunising protocol was insufficient to stimulate an eosinophilia or that the strains of the mice used had a defective eosinophil response. However, this last theory is unlikely as it has been shown that these mice are capable of generating a potent eosinophilia (Vadas, 1982). It is also posible that the times of sampling missed the response, if the rise and fall occurred in a few days.

An increase in the number of lymphocytes was equal to the neutrophilia, implying that this cell population may be more

important in generating immunity in a challenge infection than in a primary infection. As in a primary infection (see Chapter 5) there was a decline in the numbers of monocytes, except in the immunised group of SJL, in which there was a profound monocytosis. This suggests that this cell population may be important in generating immunity to a challenge infection in this strain of the host.

6.3.5 Cellular Response of Secondary Lymphoid Organs

Examination of the cells in the secondary lymphoid organs revealed a number of interesting points. There was an increased cellularity of the spleen in the unimmunised group after challenge, followed by a decline until the parasite was expelled. This pattern of events was very similar to those seen in a primary infection (see Chapter 5). In the MLN, the results were slightly different. In the immunised group, B, there was no apparent enlargement of the organ in contrast to the primary infection where the increase in cellularity of this organ was quite pronounced. However, there was a substantial increase in the cellularity of the MLN of the unimmunised group, C, after challenge, but this response had declined by the time the parasite was expelled. This suggested that in the immunised group, B, the cells that were present may have been more efficient in causing destruction of the parasite or that the response was more localised at the site of infection, the gut, and so there was no increase in the number of cells in the MLN. In the unimmunised group, C, there was quite a substantial enlargement of the MLN after challenge. Although this may have reduced the numbers of larvae developing, delayed their maturation and reduced their fecundity, it did not result in sterile immunity. However, this reaction had no effect on the longevity of the parasite and had declined by the time the parasite was actually expelled from the host, suggesting that this response may have been acting against the larval stages rather than the adult parasites and that it was this stage which was most vulnerable to attack by the immune system.

Further dissection of this response revealed that there was little change in the numbers of B cells in the spleen, although there was a rise in the numbers of these cells in the MLN. Again, there was little change in the numbers of T cells in the spleen but a slight increase in the MLN. However, the numbers of null cells rose substantially in the spleen, accounting almost entirely for the increased cellularity of this organ. The fall in the numbers of null cells in the MLN could account for the lack of cell numbers in this organ. The events which occurred in these organs were very similar to those events which took place in a primary infection when the parasite was expelled from the host (see Chapter 5).

6.3.6 Blastogenic Response of the Secondary Lymphoid Organs

When the blastogenic index of the cells from the spleen and the MLN were examined it was noted that the response was about half that obtained in a primary infection but the pattern of reactivity was very similar. This again points to the role of the spleen, which again, appears to be secondary, to the MLN in generating an immune response to the parasite. It was also noted that the events occurring in the spleen were quite clear cut, maximal responsiveness occurring at challenge and being greatest in the higher responder strains. The response declined in the unimmunised group, C, after challenge, again suggesting that the immune response was acting against the larval rather than the adult stages of the parasite. The response of the MLN cells of group C was negligible with maximum response occurring after challenge but declining when the parasite was actually expelled. However, in CBA, the response did increase when the worm was expelled indicating that although it is most likely that the worm is lost from this host through endogenous changes in the parasite, there may be some degree of immunity acting which may accelerate the expulsion of the worm or decrease its fecundity. In the immunised group, B, the story was not quite so clear cut, the greatest response of SJL occurred just before challenge and

then declined suggesting that immunity may have been acting from an early stage in this strain of the host. This pattern of response was very similar to that observed in a primary infection with this parasite. One explanation could be that the nodes enlarge early on in the infection and then when the cells are required at the site of infection i.e. the gut, they leave the organ and infiltrate the tissues surrounding the parasite, thus explaining the apparent lack of activity in the MLN. In CBA, the response was very great after challenge which could explain the lack of immunity to the challenge infection, the cells were not in the right place at the right time.

6.3.7 Response to Mitogen

In order to analyse the state of responsiveness further and to elucidate which cells were responsible for generating immunity to the parasite, their response to two mitogens was studied. The response of spleen cells to the T cell mitogen, Con A was negligible in all groups although a slight peak in activity was apparent in the immunised group, B, after challenge. The response of the MLN, on the other hand, was much higher, again implying that the role of T cells in the MLN was more important than in the spleen. These results were very similar to the response of the cells of these two organs in a primary infection (see Chapter 5). Again, this indicated that the role of the spleen was secondary to that of the cells of the MLN and that T cells may be responsible for the generation of immunity to the parasite.

6.3.8 Mucosal Mast Cell Response

From the results it was seen that although there was little or no mastocytosis in the immunised group, B, a slight mast cell response did develop in the unimmunised group C, of SJL, which was slightly greater than that observed in a primary infection in this strain. This suggested that the role of the mast cell inflammatory response had a negligible effect in the

expulsion of the parasite from an immunised host. However, it may have been more important in rejecting an infection superimposed on another. Again, it was seen that SJL responded more quickly and to a greater extent than the other two strains. As follows, it seems that a mastocytosis was not elicited nor responsible for the expulsion of the larval stages but may have been effective at rejecting the adult parasite. As discussed in Chapter 5, these results do not correlate with those seen in the study by Dehlawi, Wakelin and Behnke (1987) who demonstrated a very profound mastocytosis in male SJL challenged after a primary infection with this parasite had been expelled. However, this is probably due to the different immunisation protocol used. Instead of trying to generate immunity against the larval stages, as in the present study, mice were allowed to expel a primary infection and then challenged. In this case, the mice were challenged 4 weeks after the initial infection at which time the parasites were still present in group C. Therefore, the host was not expressing any immunity to the adult stage of the parasite, but seemed to be able to prevent establishment of a challenge infection.

Overall, it seems that a mastocytosis was elicited by the adult stage of the parasite and could be contributing to the expulsion of the parasite. As discussed earlier (Chapter 5) staining of histological sections does not reveal the functional properties of the cells. Monitoring of the RMCP II levels may reveal more substantial differences. For instance, although no cells were observed in the immunised group, analysis of RMCP II levels would reveal the presence of immature but functionally active cells or activated cells which had lost their granules, which would contribute considerably to the immune response to the parasite in both the larval and adult stages. In order to ensure that any response had not been missed it may be necessary to repeat the experiment, and take various samples earlier after challenge.

6.3.9 Antibody Response to Parasite Antigens

From these studies it was apparent that there was a considerable antibody response by both the immunised and unimmunised groups to the parasite, which appeared to be related to the degree of resistance to the infection. Interestingly, in contrast to the antibody response of animals subjected to a primary infection, the reaction to day 4 larval antigens was almost equivalent to that of the adult antigens. This implied that the protocol used, resulted in the prolonged stimulation of the immune system to both larval and adult antigens. It was also noted that the response to adult antigen achieved by all three strains of the host used, high, medium and low responders, was as great as the response of the high responder strain subjected to a primary infection. Overall, this implied that both the immunising regime and imposing a challenge infection on an existing infection prolonged the exposure of the immune system to larval antigens. This suggested that concommitant immunity may be playing an important role in resistance to reinfection with this parasite. The generation of antibodies to larval infections, may not only prevent their establishment but may also delay their development and maturation. Recognition of adult antigens appears to be correlated with expulsion of the mature stage of the parasite. Therefore, it seems that stimulation of the immune response by larval stages results in resistance to the parasite, as has been demonstrated by a number of workers (Behnke and Robinson, 1985; Behnke and Wakelin, 1977; Enriquez, Cypess and Wassom, 1988; Ey, 1988a,b; Goven and De Buysscher, 1980; Hagan, Behnke and Parish, 1981; Hosier, Sackman and Idell, 1974; Jones and Rubin, 1974; Mitchell and Munoz, 1983; van Zandt, 1973). Nevertheless, it is also apparent that in the low responder strains, despite a substantial humoral response to both adult and larval antigens, there was not only a significant number of the challenge infection surviving in the immunised group but the longevity of the parasites in the super-imposed challenge infection was considerable. This

suggested that although the antibody response may have been considerable, without the presence of a cellular response the parasite may not be expelled. In fact, it was demonstrated that the low responder strains had a poor mast cell response in comparison to the high responder strain. So, it would appear that these mice do not have an effective cellular response.

6.3.10 Concentration of Antibody Classes

When the concentration of antibody classes in the serum of the mice was analysed it was seen that there was a substantial IgG hypergammaglobulinaemia as seen in response to a primary infection (see Chapter 5). Although there was a substantial antibody production, the proportion of anti-parasite antibody was not assessed. As discussed earlier, even though there is a large anti-parasite response, this may not necessarily be protective if immune cells are not also present. Indeed, it has been illustrated (Behnke and Parish, 1979; 1981; Hagan, Behnke and Wakelin, 1981; Prowse, Mitchell, Ey and Jenkin, 1978) that both immune cells and serum are required to transfer immunity to naive hosts. In this study it was also apparent that there was a considerable IgA response. This implied that although the IgG may have been acting on the developing larval stages which are in close association with the host mucosa, IgA may be responsible for immunity to the adult stage as it is known that this form of immunoglobulin is actively secreted and responsible for intestinal immunity. The response of IgM waned quite quickly after challenge, which would be consistent with the theory of class switching.

6.3.11 Qualitative Antibody Response to Parasite Antigens

From this study, it was noted, surprisingly, that the response from both the immunised group B, and the unimmunised group C, to adult homogenate and ES and day 4 ES was negligible in all the strains. This was very difficult to explain because aliquots of the same antigen preparation was used, the same antibody dilution was used and aliquots of the same HRP-conjugated antibody were used as in the primary infection, and this was shown to be reactive on other blots. It was noted that there was no response to day 4 ES antigens and this was surprising when considering the response to them seen on the ELISA. However, the molecules that were recognised in the adult preparations were very similar to those seen in a primary infection, with molecules at 67 kDa seen both in the homogenate and in the ES and a molecule at 30.5 kDa being recognised in the homogenate. This molecule corresponds to that also recognised by antibodies directed against *N. americanus* acetylcholinesterase, thus suggesting that this may be AChE which is present in this parasite and necessay for its survival in the host. The 67 kDa molecule is one of the main constituents of the nematode cuticle and so recognition of this molecule by antibody and the subsequent cellular response to it could cause severe disruption of the cuticle and so result in the death of the parasite. Therefore, it appears that an antibody response to the parasite results in the recognition of molecules possibly essential for the survival of the parasite and therefore results in its loss, lack of establishment or delay in development.

In conclusion, it can be seen that the better the strain was at responding to the parasite in a primary infection, the higher it was at responding to challenge infections after immunisation. This was also related to the immunological parameters investigated, the better the host was at responding to the challenge the greater both the cellular and humoral response to the parasite. The reasons why some of the host strains were more capable of generating a response was not

clear, but it was proposed (Chapter 5) that the presence of an I-E class II MHC molecule on the surface of antigen-presenting cells could render the host more susceptible to the suppressive activities of the parasite.

It was noted that mice immunised with the 9-day anthelmintic abbreviated regime showed an increased response to a challenge infection in comparison to those which were subjected to a primary infection and then imposed with a challenge infection. It appears that increased exposure of the host to the larval stages, either through anthelmintic abbreviated infection or through a single infection, possibly increases the resistance to a challenge. Although there may have been considerable responses to the larval stages, these were not totally effective at expelling any adult parasites that did manage to develop. It is possible that those immune responses acting against the adult worm were somehow different from those acting against the larval stages, or that those adult parasites that did survive were capable of manipulating the immune response in some way to increase their longevity.

Overall, it was seen that prolonged exposure of the hosts immune system to larval antigens or the absence of adult parasites increased the resistance to the parasite, but the extent of this was determined by both the strain of mouse host and the mode of immunisation.

CHAPTER SEVEN

ANTIBODY RESPONSE TO SURFACE PROTEINS OF *HELIGMOSOMOIDES POLYGYRUS*

7.1 INTRODUCTION

In order to study the interactions between parasite derived molecules both in the ES and on the cuticle and the host immune system, a short investigation was initiated. Of prime interest was the antibody response of mice to a primary infection with *H. polygyrus*. This was because very few strains of the mouse host are able to expel a primary infection of this parasite. Therefore, it was thought that it would be pertinent to study the response of high, intermediate and low responder strains to molecules in the ES and on the surface of the parasite. For instance, the mice may be able to recognise functional enzymes such as AChE or proteases, or they may be able to mount a response to structural proteins such as cuticular collagens, which could lead to the disruption of the cuticle and thus expulsion of the parasite. As it is only the adult stage of the parasite that is expelled during a primary infection and because of the limited amount of material available, these were the only stages of the parasite that were studied using primary infection sera (see Chapter 5). It was thought that the high responder strain may have the ability to recognise some molecules which were 'hidden' from the low responder strain.

In later studies, in order to utilise the available material to the best advantage, it was decided to probe the CTAB supernatants and homogenates from days 1-5 p.i. with sera obtained from abbreviated infections (see Chapter 6). It was shown previously (Chapter 5) sera from primary infections did not recognise day 4 L4 ES on either ELISA or on Western blots, whereas, that from abbreviated infections recognised day 4 L4 ES on ELISA as strongly as adult ES although nothing was seen on equivalent Western blots. Thus, sera from abbreviated infections, which have had prolonged exposure to the larval stages of the parasite were thought to be more suitable for the analysis of the response to larval antigens.

7.2 RESULTS

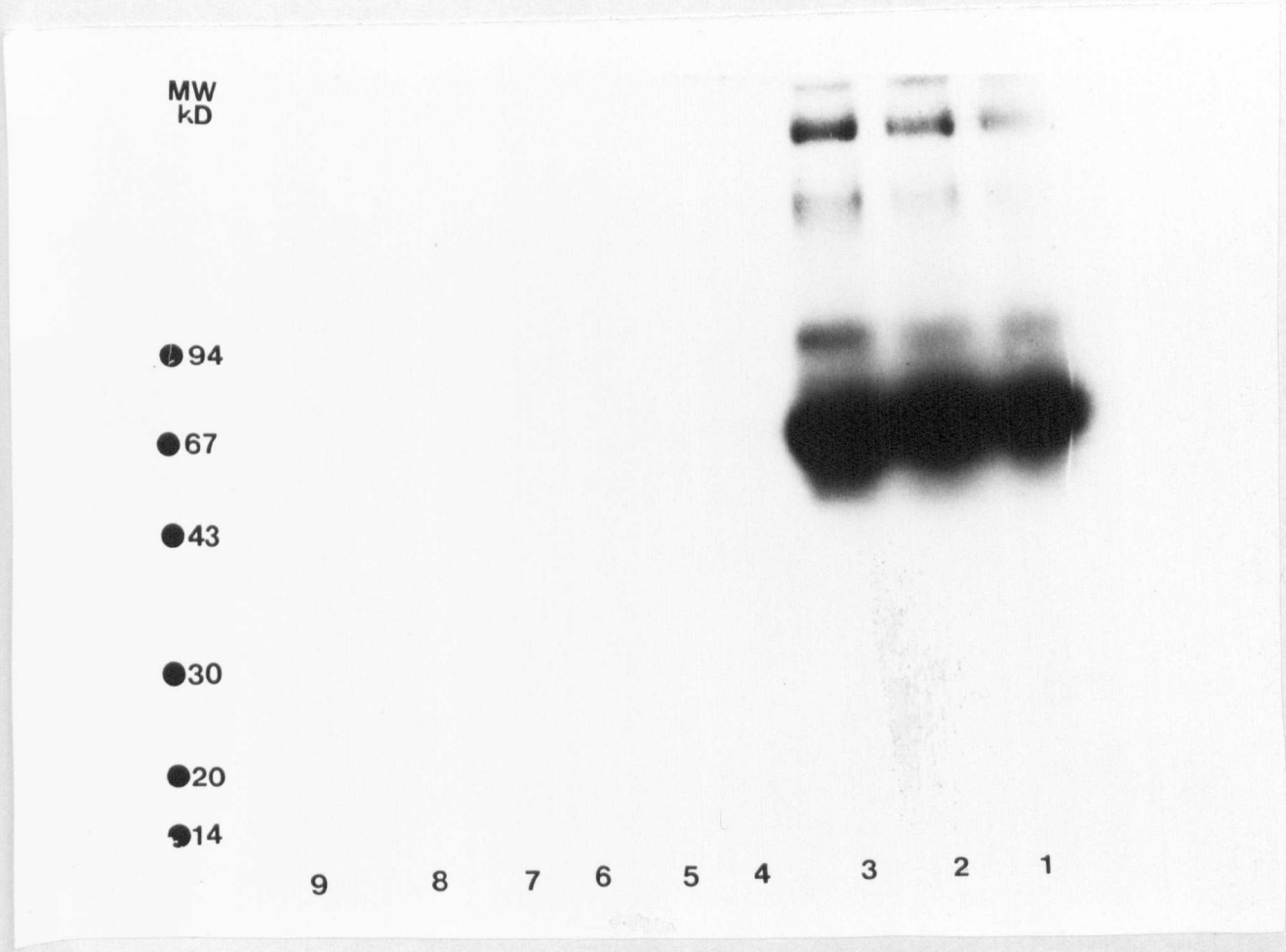
7.2.1 Recognition of Adult Antigens

Firstly, adult cuticle preparations (S1, S2 and S3) were precipitated using sera from the final bleeds, when expulsion had occurred, of SJL, BALB/c and CBA mice subjected to a primary infection with 200 L3 of *H. polygyrus*. From Fig. 7.1 it was seen that neither BALB/c nor CBA sera precipitated any molecules from any of the antigen preparations. It appears that molecules at 208, 145 and 92 kDa were precipitated more strongly in the S3 fraction than in the S2 or S1 fractions by SJL sera. The collection of molecules at 62-76 kDa were recognised very strongly by SJL in all of the preparations. It also appeared that the molecule at 47 kDa was not precipitated in any of these preparations. Interestingly, no molecules below this were seen, although it is known that there are molecules present at 20 and 15 kDa at this stage.

Figure 7.1 Immunoprecipitation of Adult Surface Proteins with Primary Infection Sera

50,000 cpm of surface labelled proteins from adult parasites extracted with PBS (S1), SDS (S2) and B-ME (S3) were precipitated with 25ul of sera from mice which had expelled a primary infection of the parasite followed by a sheep anti-mouse IgGAM. After washing, the precipitates were run under reducing conditions on a 5-20% SDS-PAGE gel and then the gels autoradiographed. The molecular weight standards are shown on the left hand side.

Lane	1	S1 vs SJL week 6 p.i.
"	2	S2 vs " " " "
"	3	S3 vs " " " "
"	4	S1 vs BALB/c week 13 p.i.
"	5	S2 vs " " " "
"	6	S3 vs " " " "
"	7	S1 vs CBA week 35 p.i.
"	8	S2 vs " " " "
"	9	S3 vs " " " "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In an attempt to reveal a response to sex specific antigens, the same sera was used to precipitate female cuticle preparations (Fig. 7.2). Unfortunately, due to the lack of male antigens the response to these could be investigated. It was seen that again, SJL recognised far more molecules than either BALB/c or CBA. In this case BALB/c precipitated molecules at 62 and 145 kDa whilst SJL precipitated all of the molecules known to be present in the preparations, particularly the 62 kDa molecule. The 47 kDa protein also appeared to be recognised in this instance.

Figure 7.2 Immunoprecipitation of Female Adult Surface Proteins with Primary Infection Sera

50,000 cpm of surface labelled proteins from female adult parasites extracted with PBS (S1), SDS (S2) and B-ME (S3) were precipitated with 25ul of sera from mice which had expelled a primary infection of the parasite followed by a sheep anti-mouse IgGAM. After washing, the precipitates were run under reducing conditions on a 5-20% SDS-PAGE gel and then the gels autoradiographed. The molecular weight standards are shown on the left hand side.

Lane	1	S1 vs SJL week 6 p.i.
"	2	S2 vs " " " "
"	3	S3 vs " " " "
"	4	S1 vs BALB/c week 13 p.i.
"	5	S2 vs " " " "
"	6	S3 vs " " " "
"	7	S1 vs CBA week 35 p.i.
"	8	S2 vs " " " "
"	9	S3 vs " " " "

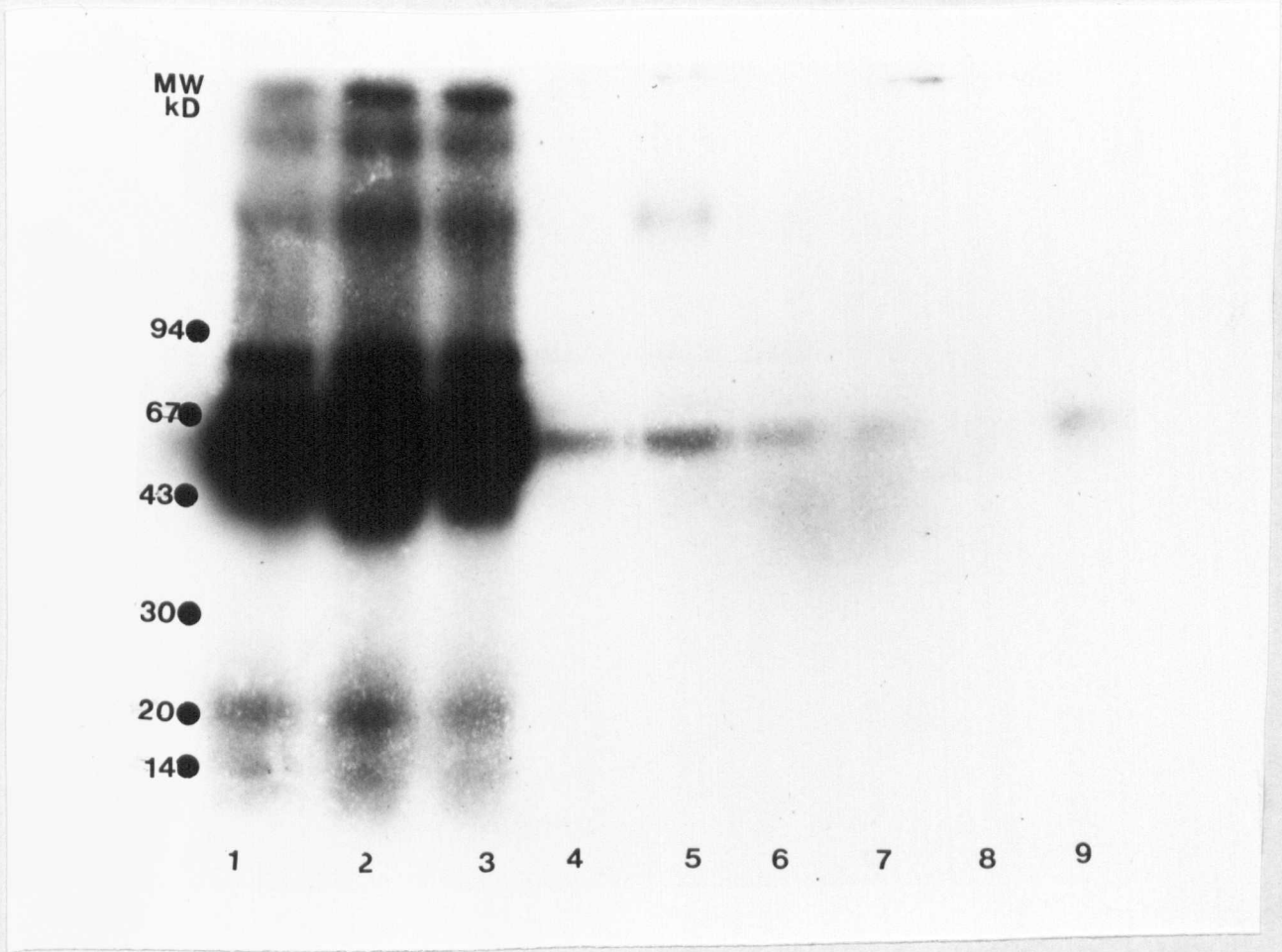
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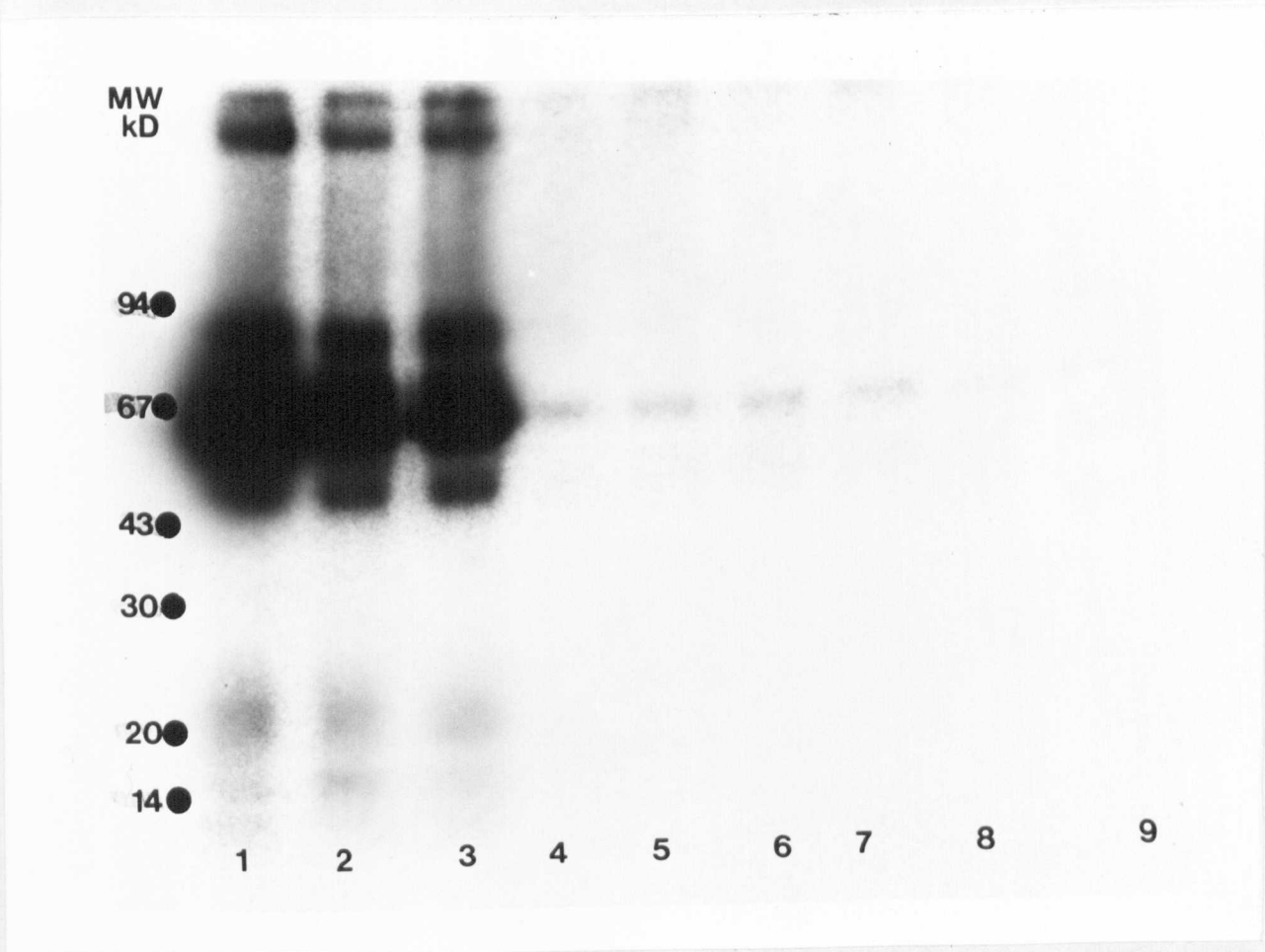
Fig. 7.3 shows the precipitations of adult worm cuticle preparations made when the cuticle was CTAB stripped prior to the labelling procedure. This was done in order to investigate the response to molecules present in the deeper zones of the cuticle. From this it was noted that again BALB/c and CBA recognised very few molecules, with only that at 62 kDa being faintly visible. SJL precipitated a wide range of molecules including those at 92, 62 and 47 kDa, molecules at 20 and 15 kDa were also slightly recognised.

Figure 7.3 Immunoprecipitation of Adult Surface Proteins Labelled after CTAB Stripping with Primary Infection Sera

50,000 cpm of surface labelled proteins from adult parasites which had been stripped with the cationic detergent cetyltrimethylammonium bromide (CTAB) prior to radio-iodination and then extracted with PBS (S1), SDS (S2) and B-ME (S3) were precipitated with 25ul of sera from mice which had expelled a primary infection of the parasite followed by a sheep anti-mouse IgGAM. After washing, the precipitates were run under reducing conditions on a 5-20% SDS-PAGE gel and then the gels autoradiographed. The molecular weight standards are shown on the left hand side.

Lane 1 S1 vs SJL week 6 p.i.
" 2 S2 vs " " " "
" 3 S3 vs " " " "
" 4 S1 vs BALB/c week 13 p.i.
" 5 S2 vs " " " "
" 6 S3 vs " " " "
" 7 S1 vs CBA week 35 p.i.
" 8 S2 vs " " " "
" 9 S3 vs " " " "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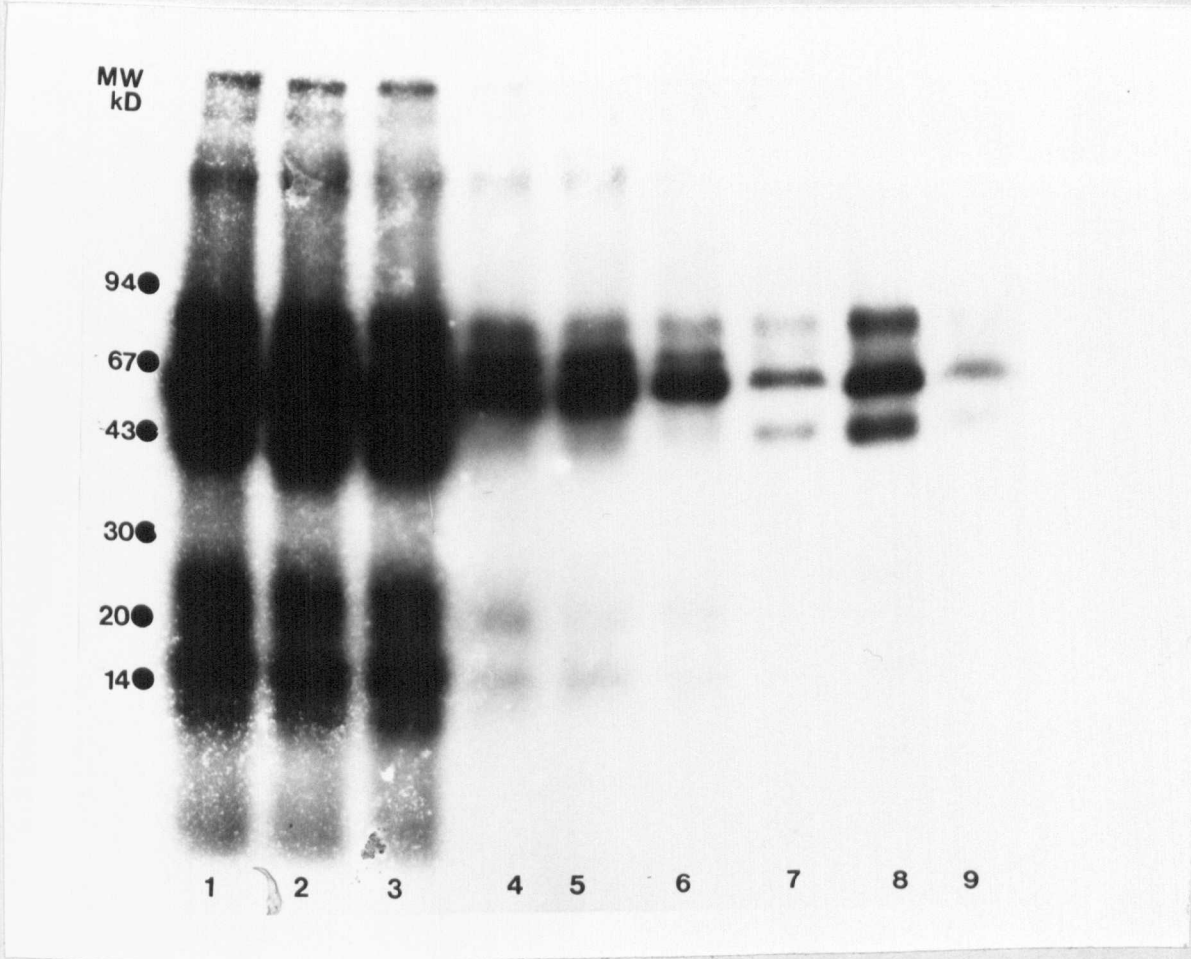
In order to characterise this response further, various extracts of the parasite cuticle were precipitated. These included the CTAB supernatant from labelled and stripped worms, and both the aqueous and detergent phases from Triton X 114 extractions. It was seen (Fig. 7.4) that all 3 sera precipitated the CTAB supernatant to an equal degree. Molecules at 145, 92, 62, 47, 20 and 15 kDa were revealed, although, the molecule at 208 kDa was not seen. In the Triton X 114 aqueous phase, SJL recognised more molecules than BALB/c or CBA but less molecules were seen than in the CTAB supernatant. The 92, 76 and 62 kDa proteins were predominant in all the precipitates but SJL additionally recognised the 145, 20 and 15 kDa molecules, the 47 kDa molecule was not recognised by any of the strains. In the detergent phase, only 3 molecules were recognised by any of the strains, with BALB/c recognising more than SJL, whilst CBA only precipitated the 62 kDa protein. The only molecules recognised by BALB/c and CBA were at 92, 62 and 47 kDa.

Figure 7.4 Immunoprecipitation of Extracts of Labelled Surface Molecules with Primary Infection Sera

50,000 cpm of surface labelled proteins from adult parasites which had been extracted from the parasite cuticle with the detergent Triton X 114 or stripped with the cationic detergent cetyltrimethylammonium bromide (CTAB) after radio-iodination, were precipitated with 25ul of sera from mice which had expelled a primary infection of the parasite followed by a sheep anti-mouse IgGAM. After washing, the precipitates were run under reducing conditions on a 5-20% SDS-PAGE gel and then the gels autoradiographed. The molecular weight standards are shown on the left hand side.

Lane 1	CTAB supernatant	vs SJL week 6 p.i.
" 2	CTAB "	vs BALB/c week 13 p.i.
" 3	CTAB "	vs CBA week 35 p.i.
" 4	Triton aqueous phase	vs SJL week 6 p.i.
" 5	" " "	vs BALB/c week 13 p.i.
" 6	" " "	vs CBA week 35 p.i.
" 7	" detergent phase	vs SJL week 6 p.i.
" 8	" " "	vs BALB/c week 13 p.i.
" 9	" " "	vs CBA week 35 p.i.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7.2.2 Recognition of Larval Antigens

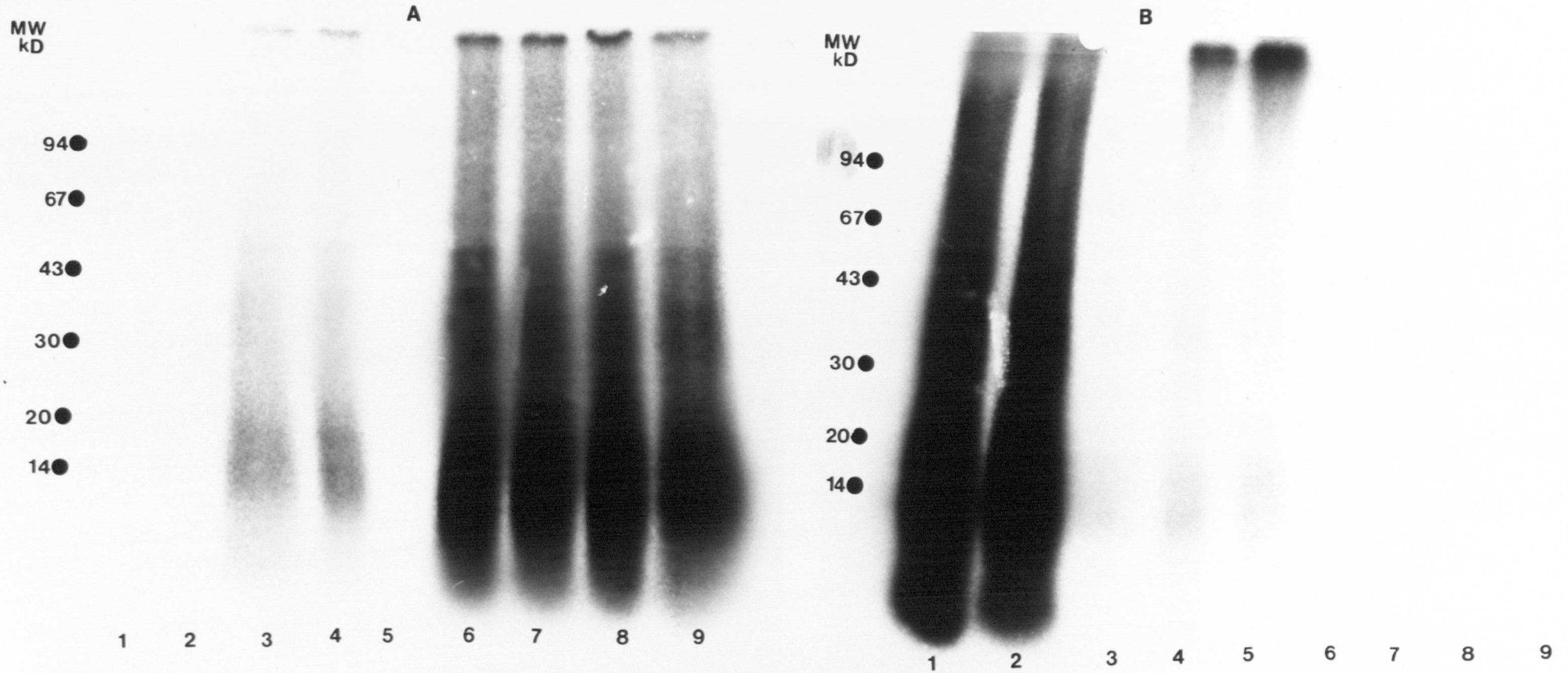
The use of sera from an abbreviated infection allowed immunogenic molecules from the larval stages of the parasite to be isolated. CTAB supernatants were used as the source of antigen as this has been previously demonstrated (see Chapter 4) to remove more than 50% of the labelled material.

Fig. 7.5a illustrates the molecules that were immunoprecipitated from days 1 and 2 using the abbreviated sera. It was seen that very little material was recognised in the day 1 preparations, SJL and BALB/c brought down molecules in the 20-15 kDa molecular weight region. A lot more material was recognised in the day 2 preparations, all three sera precipitated the entire range of molecules known to be present, particularly in the 20-15 kDa region. Fig 7.5b shows the molecules which were recognised in day 3. Only SJL and BALB/c precipitated any material from either the supernatant or the homogenate. Again, those molecules that were recognised, were concentrated in the 20-15 kDa region. Very few molecules were seen in the homogenate, with the majority of those seen being in the higher molecular weight range. CBA recognised a 62 kDa molecule in the supernatant.

Figure 7.5 Immunoprecipitation of Days 1-3 Larval Parasites with Immune Sera

50,000 cpm of surface labelled proteins from larval parasites which had been stripped from the cuticle with the cationic detergent cetyltrimethylammonium bromide (CTAB) after radio-iodination or had been extracted by boiling in SDS, were precipitated with 25ul of sera from mice which had been immunised against the parasite using the 9-day anthelmintic abbreviated infection (Robinson and Behnke, 1985), followed by a sheep anti-mouse IgGAM. After washing, the precipitates were run under reducing conditions on a 5-20% SDS-PAGE gel and then the gels autoradiographed. The molecular weight standards are shown on the left hand side.

(A)	Lane 1	Blank	
	" 2	"	
	" 3	Day 1 CTAB supernatant	vs SJL
	" 4	Day 1 " "	vs BALB/c
	" 5	Day 1 " "	vs CBA
	" 6	Day 2 " "	vs SJL
	" 7	Day 2 " "	vs BALB/c
	" 8	Day 2 " "	vs CBA
	" 9	Day 2 " "	whole antigen
(B)	Lane 1	Day 3 CTAB supernatant	vs SJL
	" 2	Day 3 " "	vs BALB/c
	" 3	Day 3 " "	vs CBA
	" 4	Day 3 Boiled Pellet	vs SJL
	" 5	Day 3 " "	vs BALB/c
	" 6	Day 3 " "	vs CBA
	" 7	Blank	
	" 8	"	
	" 9	"	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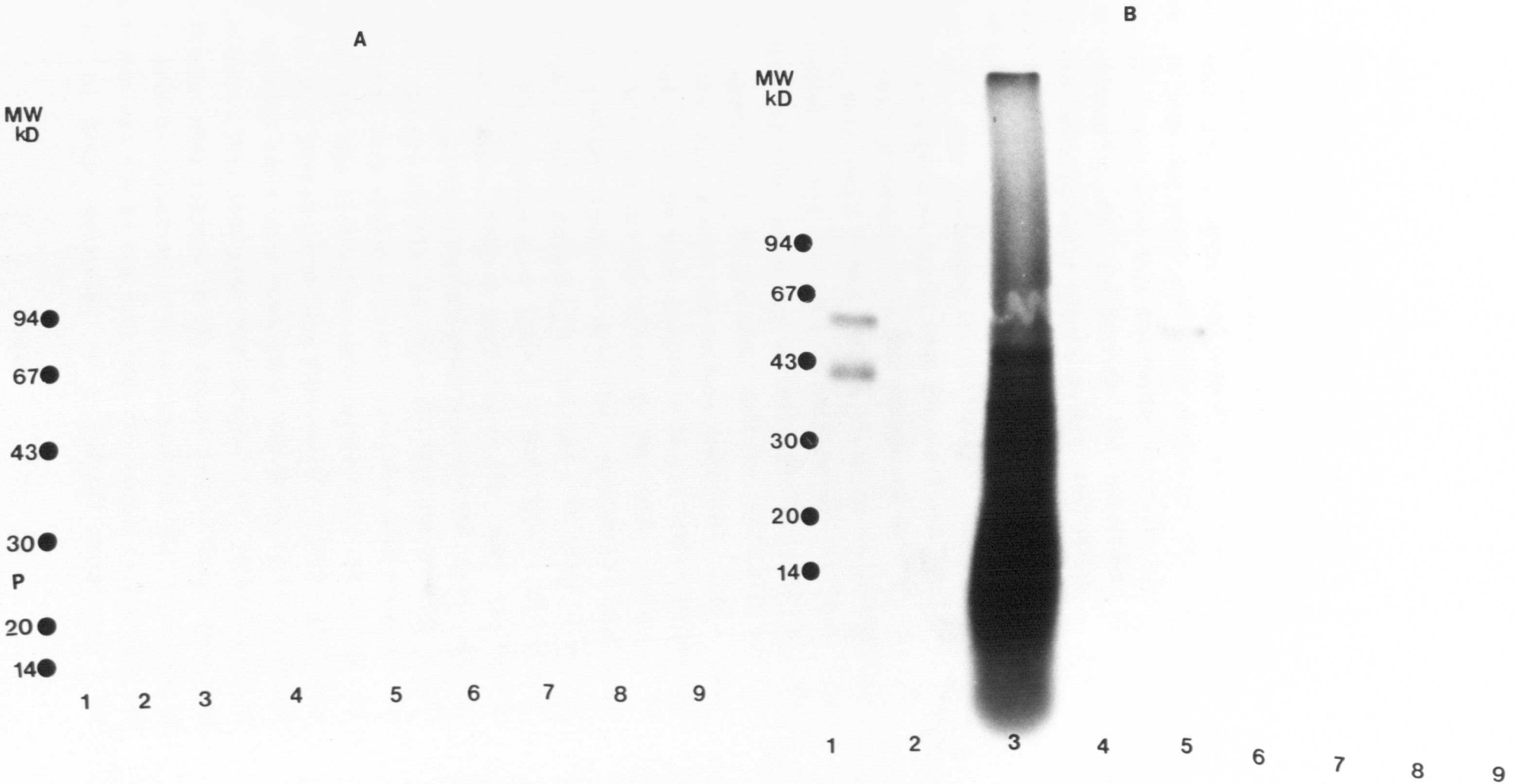
The molecules precipitated from day 4 parasites are shown in Fig. 7.6a. Unfortunately, very little was apparent on the photograph, although, on the autoradiograph it was seen that all three sera brought down equal quantities of molecules, with faint bands visible at 62, 47 and 145 kDa. In the homogenate, only SJL and BALB/c recognised any antigens and these were located at 20 kDa. From Fig 7.6b it was noted that SJL precipitated a wide range of molecules from day 5 parasites, particularly those migrating below 47 kDa. BALB/c only recognised molecules at 62 and 47 kDa whilst CBA and NMS did not precipitate any proteins. Only SJL and BALB/c recognised any molecules in the homogenate and these were located at 62 and between 20 and 15 kDa.

Figure 7.6 Immunoprecipitation of Days 4 and 5 Larval Parasites with Immune Sera

50,000 cpm of surface labelled proteins from larval parasites which had been stripped from the cuticle with the cationic detergent cetyltrimethylammonium bromide (CTAB) after radio-iodination or had been extracted by boiling in SDS, were precipitated with 25ul of sera from mice which had been immunised against the parasite using the 9-day anthelmintic abbreviated infection (Robinson and Behnke, 1985), followed by a sheep anti-mouse IgGAM. After washing, the precipitates were run under reducing conditions on a 5-20% SDS-PAGE gel and then the gels autoradiographed. The molecular weight standards are shown on the left hand side.

(A)	Lane 1	Day 4 CTAB supernatant	vs SJL
	" 2	" 4 " "	vs BALB/c
	" 3	" 4 " "	vs CBA
	" 4	" 4 " "	vs NMS
	" 5	Day 4 Boiled Pellet	vs SJL
	" 6	" 4 " "	vs BALB/c
	" 7	" 4 " "	vs CBA
	" 8	" 4 " "	vs NMS
	" 9	Blank	

(B)	Lane 1	Day 5 CTAB supernatant	vs BALB/c
	" 2	" 5 " "	vs CBA
	" 3	" 5 " "	vs SJL
	" 4	" 5 " "	vs NMS
	" 5	Day 5 Boiled Pellet	vs SJL
	" 6	" 5 " "	vs BALB/c
	" 7	" 5 " "	vs CBA
	" 8	" 5 " "	vs NMS
	" 9	Blank	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7.3 DISCUSSION

7.3.1 Recognition of Adult Antigens

From the results it was seen that the cuticle surface of *H. polygyrus* has a number immunogenic molecules which are present in both the adult and larval stages of the parasite. In general it was noted that the better the mouse host strain was at responding to, and causing the expulsion of, the parasite the more parasite molecules were recognised. There seemed to be little difference in the pattern of recognition of the various parasite cuticle preparations (S1, S2 and S3), although in some instances the S3 fraction appeared to be recognised to a greater degree than the other two fractions.

The most prominent molecules recognised were at 92, 76 and 62 kDa, with those at 208 and 145 kDa being recognised to a lesser extent. This recognition may merely reflect the proportions of the molecules on the cuticle. The 62 kDa molecule appears to be the most dominant molecule and is removed very easily with the cationic detergent CTAB. This implies that it may be more accessible to the immune system and so will be seen to a greater extent by the immune system.

The similar range of molecules recognised from each preparation is quite contrary to that seen with other parasite species. For instance, if adult *N. americanus* (Pritchard, McKean and Rogan, 1988) is subjected to the same extraction procedure, a quite different profile was noted with only 2 molecules being visible in the S3 fraction. When these preparations were probed with post-infection sera none of the molecules in the S3 fraction were recognised. When the S3 fraction was probed with sera from animals which had been immunised with adult worm homogenate, the proteins from S3 were recognised. This indicated that although the molecules were immunogenic when exposed to the immune system, they were not seen in natural infections. It was suggested that this lack of recognition was due to the fact that the immune system was not exposed to these molecules in a natural infection. The

molecules of the S3 fraction were demonstrated to be relatively small and collagenous in nature (McKean and Pritchard, 1989) but lie beneath the collagenase-insensitive epicuticle, thus rendering the molecules inaccessible to the immune response. It appeared that the same was not true for *H. polygyrus* as the molecules recognised by each serum were very similar for each preparation. This suggested that either there were no small collagenous molecules present on the cuticle of this parasite or that any that are present are not available for the labelling technique. The use of bacterial collagenase and/or RMCP II would confirm the presence of these proteins.

Even if the molecules in the S3 fraction were not collagenous, the immunoprecipitates demonstrate that an antibody response is produced which not only recognises molecules on the glycocalyx but also those located in the deeper lying zones of the cuticle. Indeed it is pertinent to note that in some cases the immune response is strongest against the S3 fraction and so it suggests that recognition of molecules in this antigen preparation could lead to the expulsion of the parasite. It was demonstrated (Adams, Monroy, East and Dobson, 1987) that immune sera recognised a 60 kDa surface protein which was not seen by either primary or naive serum, whereas, molecules at 65 and 90 kDa were seen by both primary and immune sera. This implies that recognition of a number of molecules could result in the rejection of the parasite.

When extracts from female parasites were precipitated using primary infection sera, broadly the same recognition profile was noted, although molecules at 20-15 kDa were also recognised. This suggested that these molecules may have been associated more with the female parasites than with the males. Recognition of female specific proteins could account for the reduced worm fecundity prior to parasite expulsion. The recognition of molecules at 65 and 90 kDa by both primary and immune sera in the previous study could account for the reduced parasite fecundity without expulsion occurring.

When extracts from adult parasites labelled after being stripped with the detergent CTAB were analysed using the various sera, the most significant difference observed was the absence of the 145 kDa band seen in the previous precipitates. This implied that that the stripping procedure removed this molecule completely from the surface. Interestingly, in the previous immunoprecipitates, this molecule was recognised fairly strongly in contrast to its concentration on the cuticle. Thus, it appears that this protein may be fairly immunogenic compared with some of the other molecules such as the 208 and 20-15 kDa bands.

Previous studies have shown that by removing the glycocalyx with the detergent CTAB prior to labelling (see Chapter 4) results in a wider variety of molecules being labelled to a greater extent, as a result of them being exposed to the label. Therefore, it may be expected that additional molecules may be precipitated in this fraction as more molecules would have been radio-iodinated. However, it was obvious that this was not the case. The same range of molecules was precipitated with the exception of the absence of the 145 kDa molecule. This had previously been demonstrated to be completely removed by the stipping procedure in Chapter 4.

Therefore, it appears that the molecules at 20 kDa and lower were either not immunogenic or were not naturally exposed to the immune system. Even though the number of labelled molecules at 92, 76, 62 and 47 kDa were severely depleted by this treatment it was seen that they were still precipitated to a very large extent. This suggested that these proteins may be immunodominant but they may be masking deeper lying proteins whose recognition may be potentially more damaging to the parasite and thus result in its expulsion.

Recognition of surface molecules appears to be associated with the expulsion of the parasite and a response to deeper lying proteins may accelerate the damage and rejection of the parasite from the host. Indeed an immune response against the epicuticular molecules may facilitate the removal of these molecules from the parasite surface and thus expose

structurally more important molecules whose recognition by the immune system could have serious consequences for the parasite. The poor response to the parasite surface molecules by both BALB/c and CBA could explain why expulsion of the worm takes longer in these strains. However, at the time when these sera were taken from each strain, the parasite had been or was in the process of being expelled from the host. This suggested that other factors such as the capacity of the cellular response may affect the eventual outcome of a humoral response to this parasite. In the case of CBA (see Chapter 5) it seemed most likely that the loss of the parasite was due to endogenous worm senescence rather than any immune response to the parasite. Comparing the responses of the various strains of the host at the same time points, e.g. at week 6 p.i. when SJL have expelled their worm burden but BALB/c and CBA have yet to do so, may clarify the response to the adult parasite further.

When other parasite preparations were examined, the same general pattern of antigen recognition was observed. SJL recognised more molecules and to a greater extent than BALB/c or CBA. In the CTAB supernatant, the 92, 62 and 47 kDa proteins were predominant. However, the 208 kDa molecule was not present implying that this molecule was situated in the inner layers of the cuticle, it was bound more strongly to the surface or that it was positively charged and therefore inaccessible to the cationic detergent. Another notable point was the intense recognition of the 20-15 kDa molecules in this fraction and the additional molecule at 12 kDa. This may be because these molecules are present in larger proportions in the CTAB supernatant than in the other preparations. This also implied that the molecules present on the glycocalyx of the cuticle may be more antigenic than others elsewhere and that an immune reaction to them may be sufficient to cause expulsion of the parasite. It was also noted that all three strains of the host tested, at the time when the parasite was expelled, recognised the same molecules from this fraction. It further suggested that recognition of molecules from this fraction is somehow different to that from the other fractions. This may

have been because these molecules on the glycocalyx may be modified in some way such as the presence of carbohydrate moieties which may make them more likely to be recognised by the host.

In order to assess the response to hydrophilic and hydrophobic proteins, which may be more important structural molecules, Triton X 114 extractions were precipitated. A limited range of molecules were precipitated, but this correlated with the less efficient extraction procedure. In the aqueous, hydrophilic phase, molecules at 145, 92, 76, 62, 20 and 15 kDa were precipitated with SJL reacting more strongly than BALB/c which in turn was more responsive than CBA. It was notable that the 47 kDa molecule was absent in this fraction. When the detergent phase was immunoprecipitated with the same sera, the results were quite interesting. Only three molecules were recognised by any of the sera at 92, 62 and 47 kDa. The 47 kDa molecule was not recognised in the aqueous phase. Thus from this it would appear that the 47 kDa molecule is hydrophobic whereas the 92 and 62 kDa could be amphiphilic and the 76 kDa hydrophilic. The presence of molecules in this fraction was previously attributed to contamination from the aqueous phase, however, the use of precipitates indicated that the 47 kDa molecule was indeed hydrophobic and recognised by an immune response. The fact that the 145, 76 and 20-15 kDa molecules were not present in the detergent phase further indicated the presence of hydrophilic molecules on the surface of this parasite. The large amounts of these molecules seen in the CTAB supernatant again implied that the molecules are located on the glycocalyx and readily accessible to the immune system. The presence of the 62 kDa molecule in both fractions could be the result of contamination, however this is unlikely in the light of the other molecules which were shown to be co-precipitated. It has been proposed that this band consists of a heterogenous group of molecules (Pritchard, Maizels, Behnke and Appleby, 1984) as when it is subjected to isoelectrophoresis it resolves at both pI 4.0 and pI 5.85. The revelation of hydrophobic molecules which were recognised by an

immune response to a primary infection with the parasite was very interesting. Recognition of hydrophobic molecules in the nematode cuticle not only implied that deeper lying proteins could be seen, but that possibly structurally important molecules were recognised. This suggested that these molecules may be integral surface proteins with the hydrophobic domain interacting directly with a hydrophobic anchor in the nematode cuticle (Bordier, 1981) and thus an immune response to them would clearly disrupt the parasite surface. This could then have resulted in the expulsion of the parasite from the host.

7.3.2 Response to Larval Antigens

Sera from anthelmintic truncated infections was used to investigate the response to larval antigens, between days 1 and 5 p.i. These sera were used because it was previously demonstrated (Chapters 5 and 6) that whilst sera from a primary infection from any strain of the mouse host could not recognise day 4 ES, that from abbreviated infections in the same strain of the host recognised this antigen preparation almost as strongly as ES from adult parasites. It has been widely demonstrated that whilst larval parasites can induce strong protective immunity, via a variety of immunising protocols (e.g. anthelmintic truncated infections, irradiated larval infection or injection of larvae or their products) the adult parasite can suppress an immune response, not only to homologous challenge but also to heterologous challenge (Behnke and Robinson, 1985; Behnke and Wakelin, 1977; Enriquez, Cypess and Wassom, 1988; Ey, 1988a,b; Goven and De Buysscher, 1980; Hagan, Behnke and Parish, 1981; Hosier, Sackman and Idell, 1974; Jones and Rubin, 1974; Mitchell and Munoz, 1983; van Zandt, 1973). Thus, the use of sera reactive to larval antigens may indicate which molecules are immunogenic and which molecules from the adult parasite may be immunosuppressive.

Due to the lack of material from the early stages of the infection, only a limited number of preparations could be made from the parasites. Earlier studies (see Chapter 4)

demonstrated that the majority of labelled material from these stages of the worm could be extracted using the detergent CTAB. Immunoprecipitates using adult CTAB supernatants as the antigen source (Fig. 7.4) indicated that the molecules from this fraction retained their antigenic nature and were still recognised by an antibody response. Therefore, in all the studies on the response to larval parasites, the CTAB supernatant and the homogenates of parasites subjected to this treatment were used as the antigen source. The sera used to probe these antigen preparations was taken two weeks after challenge from the three strains of mice (SJL, BALB/c and CBA) which had been immunised using the 9-day anthelmintic abbreviated infection (Robinson and Behnke, 1985).

From the results it was noted that the molecules were precipitated in a very diffuse nature and were not resolved into tight bands. This reflected the nature of the proteins which do not appear to associate into tight bands until day 4. This may be due to a number of reasons, including the presence of highly glycosylated molecules which might impede their resolution in the gel matrix, high amounts of free ^{125}I in the preparations which would contaminate the samples or the presence of labelled lipids in the samples. However despite this, it can be seen that there was a response by SJL and BALB/c to antigens located between 20 and 15 kDa. The response to day 2 antigens was considerably greater, not only to molecules at 20-15 kDa but also to those between 30 and 47 kDa. In fact, nearly all of the labelled material was precipitated even by the low responder CBA. More material was recognised in the day 3 preparations, though not by CBA. When the homogenates of day 3 parasites were precipitated very few molecules were recognised as were precipitates from day 4 CTAB supernatant and homogenate. This was probably because of the small amount of labelled material available. However, more discreet molecules were precipitated from day 4 larvae and these were located at 145, 62 and 47 kDa in the CTAB supernatant and at 20 kDa in the homogenate. Again, SJL recognised more molecules and to a greater extent than the

other two strains. In the day 5 preparations SJL recognised a number of molecules very strongly, BALB/c recognised two at 62 and 47 kDa but CBA did not recognise any molecules and this was equivalent to the recognition by NMS. Very few molecules were recognised in the homogenate and these were located at 62 and 15 kDa.

In summary, it was seen that there were a number of immunogenic molecules present on the surface of the larval stages of *H. polygyrus*. The higher the strain was at responding to the challenge infection, the greater the number of molecules recognised and the intensity with which they were precipitated. It appeared that the immunodominant molecules resolved between 15 and 20 kDa in the early larval stages and at 62 and 47 kDa in the later stages. It was interesting to note that the molecules at 62 and 47 kDa resembled those that were precipitated from adult parasites by primary infection sera. Thus, it seemed that the recognition of the 15 and 20 kDa molecules was correlated with immunity to the early L3 larval stages and that reaction to the 62 and 47 kDa proteins was associated with immunity to the later L4 larval stages and the adult parasite.

It seems possible that recognition of the low molecular weight proteins may delay the development and maturation of the early stage and mediate their destruction within the granuloma. Recognition of the higher molecular weight 62 and 47 kDa molecules may be necessary for the killing and destruction of the later larval stages and the expulsion of the parasite. Therefore, it emerges that reaction to both sets of these molecules could result in immunity to the parasite, and that the ability of the host to mount a response to the 62 and 47 kDa molecules may determine whether or not the parasite will be expelled from the host, whilst a reaction to the 15 and 20 kDa proteins may regulate the resistance to a challenge infection.

The recognition of the 62 kDa molecule on Western blots of both adult homogenate and ES by both primary and abbreviated infection sera (see Chapters 5 and 6) again implied that reaction to this molecule was crucial for the development of

immunity to the parasite. As it is also shed *in vitro* this possibly makes it more accessible to the immune system. The recognition of the 37 and 28 kDa molecules in adult ES by primary sera did not appear to be correlated to the recognition of surface molecules thus suggesting that they may be situated elsewhere in the parasite and may be involved in other functions such as modulation of the host immune system or enzymic action.

From these studies it was interesting to note that the most prominent molecules in Coomassie stained samples of parasite homogenates and ES, were those situated between 20 and 15 kDa, but these did not appear to be intensely labelled thus suggesting that they were not surface proteins. Recognition of them by the immune system was very poor both in immunoprecipitates and on blots. Despite them being the main constituents of ES, again they were not recognised by any of the sera tested. Therefore, it appeared that although they were readily accessible to the immune system i.e. they are released by the parasite, there was no reaction to them suggesting that either they are not immunogenic or that they may be involved in modulation of the hosts immune response in some way. Indeed, it has been demonstrated by a number of authors (Losson Lloyd and Soulsby, 1985; Reed, Dehlawi and Wakelin, 1988; Monroy, Dobson and Adams, 1989) that low molecular weight components of less than 26 kDa were capable of inhibiting the proliferation of mitogen stimulated mouse spleen lymphocytes. Thus, if an immune response could be generated against these molecules, the parasite would no longer be able to manipulate the hosts immune response and thus an effective response could be generated to the parasite and expulsion could occur.

Immunoprecipitates of preparations of adult parasites revealed that there were molecules of less than 26 kDa that were recognised by mice which had expelled the parasite, but not by the low responder strains. Indeed, it was also noted that they were precipitated in high concentrations from CTAB supernatants thus suggesting that they were accessible to the

immune system and therefore may be likely candidates for the immunomodulatory factor(s).

As discussed earlier, other authors have reported the presence of stage specific antigens on the surface of *H. polygyrus*. The dominant molecules appeared to be those at 60, 65 and 90 kDa (Pritchard et al, 1984; Adams, Monroy and Dobson, 1989) which possibly correspond to the 62 and 92 kDa molecules seen in this study. Indeed, the 60 kDa molecule was reported to be only recognised by immune sera and not by primary infection sera. As it was also found in ES, there was some debate as to whether it was shed from the cuticle into the medium or if it was absorbed onto the surface of the parasite from the ES. This has been suggested to explain the presence of ES antigens on the surface of *T. spiralis* (Silberstein and Despommier, 1985a,b). However, it was demonstrated that the molecules did not cross-react with proteins from the stichosome, the main secretory organ of this parasite (Parkhouse, Philipp and Ogilvie, 1981) and so it was proposed that these molecules were released from the parasite cuticle.

It appears that this may also be the case for *H. polygyrus* as it was shown (Monroy, East, Dobson and Adams, 1989) that the 60 kDa molecules existed as a trimer on the cuticle of the parasite and they concluded that it was cleaved off the surface in some way and secreted into the culture medium.

Pritchard, Maizels, Behnke and Appleby (1984) demonstrated the presence of a 16 kDa molecule on the surface of day 6 larvae and Adams, Monroy, East and Dobson (1988) illustrated the presence of a 60 kDa adult specific molecule and a 20 kDa L3 specific protein, with molecules at 33, 45 and 50 kDa common to all stages. It is possible that the 16 and 20 kDa molecules were the same and equivalent of the 15 kDa and 20 kDa molecules demonstrated in this study.

Although there have been few successful vaccinations using surface antigens (Philipp and Rumjaneck, 1984) suggesting that a response to these molecules is ineffective at causing expulsion of gastrointestinal parasites, several authors have

reported a number of effects on parasites. For instance, the infectivity of *T. spiralis* new-born larvae is significantly reduced when they are exposed to an IgG monoclonal antibody directed against a 64 kDa surface protein (Ortega-Pierres, Chayen, Clarke and Parkhouse, 1984). Monoclonal antibodies against surface determinants of *S. mansoni* schistosomula have been shown to mediate *in vitro* killing of larvae and passive protection against *in vivo* challenge (Grzych, Capron, Bazin and Capron, 1982). It has also been demonstrated (Monroy, Brindley and Dobson, 1988) that soluble (homogenate supernatant) and particulate (cuticular proteins) antigens have different abilities to immunise animals. When administered separately the effects were minimal, however, when used together they appeared to act synergistically. It was suggested (Monroy and Dobson, 1987) that this was because the cuticular components were acting as adjuvants. In fact, it has been shown (Nishimura, Nishimura, Nishi, Numata, Tone, Tokura and Azuma, 1985) that chitin derivatives, which are known to be present in cuticles, have adjuvant properties.

The fact that some major surface proteins demonstrated to be present were not recognised by immune sera in a number of parasitic infections has been attributed to the presence of host albumin absorbed onto the parasite surface. This has been illustrated for *Litmosomoides carnii*, adult *W. bancrofti* and the microfilariae of *O. gibsoni* (Maizels, Philipp, Dasgupta and Partona, 1983; Mitchell et al, 1982). Host glycolipids have also been found on the surface of *W. bancrofti*, *Loa loa* microfilariae and *A. lumbricoides* (Ridley and Hedge, 1977; Soulsby and Coombs, 1959). The importance of the presence of these molecules on the parasite surface is not fully understood, but it is thought that they may help in the evasion of the host immune responses by `masking' potentially immunogenic molecules. However, in the case of *H. polygyrus* infections it appears that this is not the case. This is because the molecule that resolves at the same weight as host albumin, and which labels very intensely, is recognised by both primary and abbreviated infection sera. This molecules would

not be recognised if it were of host origin.

In conclusion, it was noted that the proteins on the surface of *H. polygyrus* were immunogenic. It was likely that an antibody response to them could have mediated the expulsion of the adult parasite. This response may also have retarded the infection and development of the larval stages of a challenge infection. Nevertheless, it was not known what the functional involvement of these molecules was but it was proposed that they were either structural molecules or that they could have been involved in the modulation of the hosts immune response. Recognition of surface molecules may not necessarily damage the parasite as the turnover of some of these molecules may be very rapid. Therefore, even if antibody binds to surface molecules it may be ineffective in causing damage if they are sloughed off before the effector arms of the immune system could be activated.

CHAPTER EIGHT

SUMMARY DISCUSSION

8.1 SUMMARY DISCUSSION

Heligmosomoides polygyrus is a chronic gastrointestinal trichostrongyle nematode parasite of mice. It is known to induce a chronic primary infection in most strains of the mouse host and so it is often used to study the mechanisms of the depression of immunity which allow the parasite to survive for up to 8 months in a naive host. Immunisation usually involves the prolonged exposure of the immune system to the larvae or their products, whilst attempted immunisation with live adult worms or their products is largely unsuccessful and may even result in the depression of both homologous and heterologous immunity (Ali and Behnke, 1983; 1984; Behnke, Ali and Pritchard, 1983; Cayzer and Dobson, 1983; Colwell and Wescott, 1973; Crawford, Behnke and Pritchard, 1989; Dehlawi and Wakelin, 1988; Jenkins and Behnke, 1977;Losson, Lloyd and Soulsby, 1985; Monroy, Dobson and Adams, 1989; Price and Turner, 1983a,b,c; 1987; Pritchard, Ali and Behnke, 1984; Shimp, Crandall and Crandall, 1975).

The aim of this work was to investigate some of the factors which may be responsible for the generation of an immune response by the larval stages and/or immunomodulation by the adult parasites. This study was approached from two angles, firstly, the molecular characteristics of the various stages of the parasite were analysed in order to determine if any alteration in the biochemical properties of the parasite could be related to the change in their immunogenicity. Secondly, the immune response to both a primary and an immunising infection of the parasite in three strains of mice with known responsiveness to the parasite was defined. The immune reaction to the parasite was characterised by investigating a number of parameters including, the humoral response (antibody concentrations and response to parasite antigens) and cellular response (peripheral blood changes, changes in cellularity and cellular responses in the secondary lymphoid organs and the mast cell response in the gut).

8.2 FUNCTIONAL MOLECULES OF *H.POLYGYRUS*

The investigations of the functional molecules revealed that acetylcholinesterase (AChE) produced by the parasite could be playing an important role in stage specific immunity. It has long been proposed that parasite AChE has an important association with the longevity of nematode infections although its precise function has yet to be elucidated. There have been a number of mechanisms proposed as to its action in parasite infections and these have included, acting as a 'biological holdfast' causing local anaesthesia of the host intestine and thus inhibiting peristalsis, inhibition of mucus secretion, parasite neurotransmission and damping down of the immune response. The importance of each has yet to be defined, however, it is likely that a combination of these factors occurs and thus results in the overall delayed expulsion of the parasite (Bird, 1966; Holmes, Michaeli and Fudenburg, 1973; Johnson, Smith, Torres and Blalock, 1982; Jones and Ogilvie, 1972; Omar and Kuhlow, 1977; Plaut, 1987; Philipp, 1984; Rathaur, Robertson, Selkirk and Maizels, 1987; Rhoads, 1981; Specian and Neutra, 1980; 1982).

The maximum production of AChE was by the fourth larval stage of the parasite and it was suggested that this was because at this developmental stage the parasite is encysted in the mucosa and so is probably causing maximum stimulation of the immune system at a time when the larva is at its most vulnerable to the damaging effects of the effector cells. As an adult the parasite still produced significant quantities of the enzyme, although not in such high proportions of the ES as the larvae. It was suggested that although AChE may be of primary importance to the larval stages of the parasite, it is still required by the adult worm, though not to such a degree. Although the enzyme may play some role in the immunomodulation of the host by the parasite, it is probably not primarily responsible for the depression of both homologous and heterologous immunity of the host by the adult stages of the parasite, as this stage produces less of the enzyme than

immunostimulatory stages. It was noted that a molecule at the same weight as that recognised by a rabbit immunised against purified *N. americanus* AChE was recognised on blots by mice which had expelled the parasite from a primary infection or that were immune to challenge after an anthelmintic abbreviated infection. However it was not recognised by those still experiencing a chronic infection or not immune to challenge after an immunising infection. It was speculated that if it was AChE tthat was being responded to in these mice it could be playing an important role in the suppression of the immune response although it may not be prime candidate for the immunomodulatory factor of the parasite. In order to confirm the role of AChE in this parasite infection a number of further studies could be done. Firstly, the molecule could be purified on an edrophonium column and sera from mice immune to the parasites used to probe blots. Finally, antibodies against purified AChE could be used to immunise mice or to assess the affect of worm viability *in vitro*.

The study of the proteases produced by the parasites showed a different pattern of enzyme production when compared to the AChE secretion. The maximum production of these enzymes (both quantitively and qualitatively) occurred in the third larval stages of the parasite, although there was some activity produced by the L4 and adult. It was speculated that the enzymes produced could be mainly required for the exsheathment of the infective L3 and the penetration of the mucosa by this stage. The production of enzymes by the latter stages was not only smaller in quantity but also of a more restricted molecular weight profile. This possibly suggested that the escape of the adult parasite from the mucosa into the intestinal lumen was mainly mediated by mechanical action of the worm, although proteases may also have played some part in the dissolution of the host mucosa. It was proposed that the possible role of these enzymes in the L4 and adult stages was in feeding and it was suggested that they may also be involved, to some degree, in the immunomodulation of the host. It has previously been demonstrated that some parasite proteases are

capable of cleaving the Fc portion of host antibodies whilst others have been implicated in the cleavage of IL-2 and/or its receptor, the action of both would clearly have profound effects on the host immune system thus enabling a chronic infection to survive (Plaut, 1983; Chapman and Mitchell, 1982; Auriault, Ouassi, Torpier, Elsen and Capron, 1981; Leid, Suquet, Bouwer and Hinrichs, 1986).

8.3 STAGE SPECIFIC EXPRESSION OF SURFACE MOLECULES OF *H. POLYGYRUS* AND THE IMMUNE RESPONSE TO THEM

In an attempt to analyse which molecules present on the surface of the various developmental stages of the parasite could be responsible for the immunomodulation/stimulation of the host, the surfaces of the parasites from L3 to day 10 p.i. were radio-iodinated, and the labelled molecules extracted using a number of increasingly stringent procedures. It was obvious that molecules present from days 1-3 post infection were of a more restricted profile than the latter stages, and the moult from L3 to L4 was accompanied by the appearance of a 62 kDa molecule, which persisted for the rest of the the life of the nematode. As the parasite developed further, molecules appeared at 208, 145, 92, 76 and 20 kDa, ans appeared to accompany the moult from L4 to adult on day 7. By day 8 the surface protein profile was very complex resembling that of a mature worm with little or no change as the parasite aged.

The presence of fewer molecules in the more antigenic larval stages suggested that either the unique molecules in the L3 were causing stimulation of the immune system, or that the molecules produced later were either not immunogenic or were actively suppressing the immune response. The increased complexity of the surface of the parasite suggested that the cuticular molecules may be involved in the evasion of immunity by the nematode. There are four possible mechanisms by which this could occur, firstly, by being constantly and rapidly removed damage caused to the parasite by the action of bound

antibodies is minimal as the molecules are continually sloughed off, secondly, by masking potentially more immunogenic and important structural molecules, thirdly, by being the molecules responsible for the immunomodulation of the host and lastly, by being molecules such as surface bound proteases which cleave off antibodies and complement which may have bound to the parasite surface and thus prevent cell and complement mediated damage to the parasite surface. It is possible that a combination of all or a number of these factors is occurring. This could not only subvert damage to the parasite but may also depress the immune response to homologous and unrelated antigens.

The use of various extraction procedures revealed that the majority of molecules present on the cuticle of the various stages of the parasite could be easily removed both with PBS and the cationic detergent cetyltrimethylammonium bromide (CTAB) and thus suggested that these molecules were readily accessible to the host immune system. It was also demonstrated that there were no small collagenous molecules present on the nematode cuticle as has been illustrated for the human hookworm, *Necator americanus* (Pritchard, McKean and Rogan, 1989). The molecules present in the PBS, SDS and 2-ME soluble fractions were all very similar which implied that all of the molecules were accessible and potentially immunogenic or important molecules were not 'masked' by other less immunogenic proteins. However, it was interesting to note that when the various preparations were immunoprecipitated using sera from mice which had expelled a primary infection or which had been immunised using an anthelmintic abbreviated infection, a number of molecules were only recognised by those but not by others which were still experiencing an infection of adult parasites. This implied that the molecules were only recognised once a competent immune response against the parasite had been mounted and not whilst they were in the gut possibly causing antigenic stimulation but negating this through the production of immunomodulatory factors. It was implied that although the molecules were immunogenic, they were not available to the

immune system. This may either have been because the molecules were previously masked by non-immunogenic molecules or they were suppressive factors which were being produced by the parasite and were preventing the immune response from acting to its full extent. Once the factor has been neutralised by the host either through an immune response or some other method, the parasite cuticular molecules can be recognised, thus possibly causing structural damage of the cuticle and ultimately resulting in the expulsion of the parasite.

8.4 IMMUNE RESPONSE TO A PRIMARY INFECTION OF *H. POLYGYRUS*

The immunological response of three inbred strains of the mouse host, SJL, a high responder, BALB/c, an intermediate responder and CBA, a low responder, to a chronic primary infection with *H. polygyrus* revealed a number of points. It was noted that in each case, the better the strain was at causing the expulsion of the parasite, the greater was the immune response as assessed through the use of a number of criteria including WBC, differential cell counts, the B and T cellularity of the secondary lymphoid organs, the response of these cells to mitogens, the mucosal mast cell response and both a quantitative and qualitative antibody response to parasite antigens. Although it was obvious that an overall higher immune reactivity was causing the expulsion of the chronic infection in the high responder strains the exact mechanism by which this occurred was not defined. It was postulated that the phenotype of the class II MHC molecule I-E could have been influencing the response to and/or affected by various parasite products as has been proposed for *T. spiralis* infection in mice (Wassom, Krco and David, 1987).

The loss of parasites from CBA, a low responder strain did not appear to be related to the induction of a large immune response against the parasite and so it was suggested that the loss was mainly due to parasite senescence. Indeed, it was noticeable that not only were the few remaining parasites

stunted in size, but that they had also lost their bright red colouration common to healthy worms and were a dull brown colour. Analysis of homogenates of `old' worms revealed that most of the molecules present from day 20 were intact but there was a notable absence of two molecules at 19 and 17 kDa. These molecules along with 3 others at 18, 15 and 13 kDa take up the bulk of protein present in ES and homogenates and are present in all of the life cycle stages of the parasite, however, despite their strong concentration they were not radio-labelled nor recognised by immune sera on Western blots. This suggests that perhaps they are not on the surface of the parasite and not easily accessible to the labelling technique and/or they are not immunogenic. It was proposed that the lack of immunogenicity could be due to lack of exposure to the immune system, however, this is unlikely as they are released in large amounts in culture. They may not stimulate an immune response due to the poor immunogenicity of the molecular conformation. They may even be immunomodulatory and so would suppress an immune response both to themselves and heterologous stimuli. Many authors have described molecules of low molecular weight which are present in both larval and adult parasites which cause a depression of the immune response (Losson, Lloyd and Soulsby, 1985; Monroy, Dobson and Adams, 1989). Therefore, it can be appreciated that a depletion of molecules in this molecular weight range from `old' parasites, concurrent with a loss from a previously unresponsive host suggests that they must be crucial to parasite survival and may even be the factors responsible for the immunosuppression of the host.

The exact mechanism of the parasite induced immunosuppression has yet to be elucidated but it seems likely that some parasite derived product could be inhibiting the action of IL-2 either through the production of an IL-2 inhibitor or by a protease-mediated destruction of IL-2 or its receptor or by the inhibition or blocking of the receptor. All or a combination of these factors would lead to a depression of the immune response to both homologous and heterologous antigens.

8.5 IMMUNE RESPONSE TO AN IMMUNISING INFECTION OF *H. POLYGYRUS*

In an attempt to try to elucidate the immune response to the parasite further the three strains of mice previously used were immunised using an anthelmintic abbreviated infection which has been shown to be efficient at discriminating between medium and low responder strains of mice (Behnke and Robinson, 1985). From these studies it was obvious that although a potent immune response was occurring which prevented the establishment and/or development of the parasite, this had little effect on any parasites which had managed to mature to the adult stage. This population managed to survive for the same length of time or longer as those in a primary infection. It was also noted that some strains of mice were more responsive than others to the immunising regime, the higher responder strains showing a greater degree of immunity to challenge. The higher responders had a greater degree of immunity at challenge than the low responders. Although there was a considerable immune response acting at the time of challenge, which restricted the number of larvae developing this did not seem to affect any adult worms that had managed to mature. It was shown that there were both larval and adult specific antibodies on ELISA but not on Western blots, in contrast to the response to a primary infection where only antibodies to the adult stage were found. Therefore, it seems that responses against the larval stage leave the adult parasite unaffected. It was proposed that prolonged exposure of the immune system to larval products, in the absence of the adult worm promotes the recognition and destruction of molecules from this stage and ultimately results in their death. However, some parasites are unaffected and on reaching maturity are not only able to survive but can also depress the immune response to heterologous challenge. The general conclusions drawn from this study were that although a profound response to larvae could be generated in all of the strains of the mouse host, this did not appear to severely affect the longevity of the surviving adult parasites. The reasons for

this were attributed to unique immunogenic molecules present on the larval stage and/or the production of immunomodulatory factors by the adult parasite. It appeared that both of these hypotheses may be responsible as it was shown that there were unique larval antigens present on the parasite cuticle recognised by challenge sera and it is known that adult parasites are capable of suppressing an immune response both to homologous and unrelated antigens.

From the studies of the immune response to an immunising or primary infection of *H. polygyrus* it was apparent that the greater any of the immunological parameters studied, the better the strain was at responding to the infection. The exact mechanisms controlling this were not elucidated but it was suggested that the possession of I-E molecules rendered the strain more susceptible to the immunosuppressive activities of the parasite and that additional MHC molecules may modify this effect making the mouse more or less susceptible to the suppressive activities of the nematode (Wassom, Krco and David, 1987).

8.6 THE CONTROL OF IMMUNE SUPPRESSION

The control of immune suppression has been extensively studied for a number of years. Immune suppression (Is) genes have been defined by analogy with immune response (Ir) genes, they determine whether, and the extent to which, active suppression is produced. This active suppression means that non-responsiveness due to the presence of Is genes differs from the non-responsiveness due to the absence of Ir genes and thus is inherited in a different pattern. Ir gene effects are manifested in a dominant or co-dominant manner, rendering an F1 (r x n-r) cross a responder, in contrast, Is genes tend to produce dominant non-responsiveness (Oliveira and Mitchison, 1989).

It would be to the advantage of the pathogen to display particular epitopes (T suppressor epitopes) which, having a tendency to associate with and therefore be seen in the context of, certain class II molecules (I-E) acting as Is gene products, would activate the suppressor cell system. There are numerous examples of this class II restricted suppression in a wide range of different systems thus indicating the extent of this phenomenon (Baxevanis, Nagy and Klein, 1981; Milich, Leroux-Roels, Louie and Chisari, 1984; Arif, Mitchison and Zuckerman, 1988; Levich, Weigle and Parks, 1984; Oliveira, Blackwell, Virchis and Axelrod, 1985; Araneo and Yowell, 1985; Fairchild and Moorhead, 1986; Waltenbaugh, Sun and Lei, 1986; Wassom, Krco and David, 1987; Nishimoto, Kikutani, Yamamura and Kishimoto, 1987; Blackwell and Roberts, 1987; Mowat, Lamont and Bruce, 1987; Nanda, 1989; Miyashita, Moriwaki and Migita, 1989).

There appears to be two schools of thought about the inheritance of resistance to *H. polygyrus*. The first implies that resistance is inherited dominantly (Prowse and Mitchell, 1980; Enriquez, Zidian and Cypess, 1988; Robinson, Wahid and Behnke, 1989). Here, mice can be divided into differing levels of resistance, mice with **s** (A.SW and SJL) or **q** (BUB/Bn, DBA/1, B10.Q, NIH and SWR) haplotypes develop resistance after a primary infection with the parasite. Those with an **a** or **d** haplotype (A, A/He, BALB/c and DBA/2) are immune after 2 infections, whilst those with **b** (C57BL/6) or **k** (AKR, C3H/He, C3HeB/Fe, CBA, RF, B10.BR and B10.K) haplotypes develop no resistance with any number of immunising infections or regimes. Mice can be seen to inherit resistance dominantly and susceptibility recessively, for instance, SJL x CBA (s x k) and SJL x F1 give mice which are resistant. Gene complementation is also thought to be a feature of immunity to this infection as it has been shown that if SJL (s) are crossed with SWR (q) the offspring develop greater resistance to the parasite than would have been observed with either parent (Wahid and Behnke, unpublished observations), this implies that resistance may have been inherited co-dominantly in this case. It is proposed

that there are 2 MHC genes involved in the resistance to this infection, one at the D end and the other mapping left of E-alpha (Enriquez, Zidian and Cypess, 1988), but it is also possible that background genes could be modulating the expression of resistance as mice of the same haplotype but with different genetic backgrounds showed varying degrees of immunity to the parasite as has been demonstrated for *Trichuris muris* in mice (Else and Wakelin, 1988).

The influence of MHC as well as non-MHC genes may be modulated by the number of exposures to parasite antigens (Jacobsen, Brooks and Cypess, 1982), infective dose (Sitepu, Dobson and Brindley, 1985) as well as the presence or absence of adult worms (Behnke and Robinson, 1985; Enriquez, Zidian and Cypess, 1988). The target stages of the parasite may also vary according to the strain of the mouse host and the immunising regime used. Innate immunity to the parasite may also be genetically controlled at both the level of parasite infectivity and the level of growth and reproduction of the parasite (Brindley and Dobson, 1983).

It has also been postulated that resistance is modulated by the presence of Y chromosomes (Enriquez, Zidian and Cypess, 1988) since male animals are more susceptible to the infection than females. However, it is more likely that the presence or absence of the various sex hormones is having a modulatory effect on the immune response (Dobson and Owen, 1978).

The alternative concept is that resistance is inherited recessively and Is genes are dominant in the control of immunity to the parasite. Wassom, Krco and David (1987) demonstrated that resistance to *T. spiralis* was inherited in a recessive manner and that this was due to the presence of the I-E class II MHC molecule on the surface of antigen-presenting cells. It was proposed that the presence of this molecule made the host more susceptible to the suppressive activities of the parasite as recognition of antigens in association with this molecule are thought to elicit T suppressor rather than helper cells (see Chapter 5). It is possible that a similar mechanism is also operating in a *H. polygyrus* infection.

From these studies it would appear that overall, there is more than one mechanism operating in the control of resistance to *H. polygyrus*. Referring to the initial concept of Ir and Is genes it emerges that, in this case, both sets of genes are operating. Firstly, when resistance is inherited Ir genes must be operating and dominant to Is genes and secondly, when susceptibility is inherited the Is genes must be operating and dominant to the Ir genes. Therefore it seems that there is a balance between the expression of Ir and Is genes which may be dependent on which are present, in what proportions and the influence of accompanying background genes. Thus, the parasite may be more or less able to initiate or suppress an immune response and some hosts may be more or less susceptible to the suppressive activities of the parasite depending on the balance of these two sets of genes.

It has been proposed that chronic infections may also be able to survive through the induction of immunological tolerance. It has been postulated (DeFranco, 1989) that mice expressing the I-E class II MHC molecule could be readily induced to tolerise antigens. The implication was that clonal anergy induced in mature T cells resulted in the unresponsiveness of helper T cell clones. The helper T cell recognition of antigen plus MHC on cells may also lead to unresponsiveness because the antigen-presenting cells are not delivering the appropriate second signal to the helper T cells.

To conclude, the use of inbred mouse strains, with different degrees of susceptibility to the parasite allowed for a detailed investigation of some of the mechanisms controlling a chronic nematode infection. The response to larval proteins and the generation of acquired immunity to the parasite was studied by using anthelmintic abbreviated infections. In general, it was noted that the strains of the host with the greatest resistance to the parasite were able to produce a swifter and more substantial response to the parasite, but were not able to produce any unique response or recognise any additional parasite molecules. However, the exact mechanism controlling susceptibility to the parasite was not determined.

8.7 FURTHER WORK

Although this series of studies answered some of the questions posed on the generation of a chronic nematode infection, many remain unanswered, additional investigations may elucidate the host:parasite relationship further.

Purification of AChE could reveal its true importance in this infection. Immunisation of mice with AChE either purified from ES or as a cloned product, could reveal if it is more important to the adult or larval parasite i.e. is it more important in the establishment and development of the larvae or is its role more concerned with the immunodepression of the host by the adult. Studies of the proportion of the isoenzymes present in the ES of larval and adult parasites and the alteration in proportions in parasites undergoing immune attack may also provide some insight as to the importance to the parasite and the generation of a chronic infection as for *N. brasiliensis* (Ogilvie, 1972; Sanderson, Jenkins and Phillipson, 1972).

It was obvious that proteases played an important role in the establishment and the development of the parasite. Further characterisation could assess the degree to which their stage specific production is related to the environment and life cycle of the parasite. The use of various inhibitors, substrates and pH's could clarify their nature, whilst the use of various extraction procedures could lead to their purification and use in immunisation experiments. The study of their importance in immunity to both a primary and challenge infection may help to ascertain their role in the infection and the contribution to the immunogenicity or immunomodulation of the various stages of the parasite.

Further isolation and characterisation of the molecules present on the cuticle of the various stages of the parasite and the immune response to them in both primary and challenge infections would undoubtedly reveal which were of crucial importance to the parasite. The use of bacterial collagenases and mast cell protease would reveal which, if any, of the

molecules were collagenous and so are more susceptible to damage by an effector cell response. The use of other proteases would also characterise their nature further and so help to form some idea of the structure of the cuticle, the importance of the various molecular components and the effect of an immune response to them. Immunisation of mice with purified surface antigens from the various stages to give some idea of the relative importance to the parasite and their possible functions i.e are there proteases which may induce protection or are there immunomodulatory molecules, the recognition of either of these would have serious consequences for parasite survival.

The use of primary infections was very useful for the study of the factors controlling the generation of a chronic nematode infection, further work should include the role of cytokines in immunity to the parasite. The study of IL-1, IL-2, IL-3, IL-4, tumour necrosis factor (TNF) and gamma-interferon throughout a chronic primary infection in various strains of mice should help to explain what it is that the parasite immunomodulatory factors are manipulating and how chronic infections manage to survive. Injection of these purified cytokines or antibodies against them may accelerate the rejection of the parasite or extend the chronicity of the infection, again, which would explain which part of the immune response is being affected by the parasite and possibly start to explain the ways in which the host can overcome these effects.

Although the use of immunising infections gave some information as to the factors controlling acquired resistance to the parasite, because of the complex nature of the immunising protocol certain effects could not be directly attributed to a single causative factor. The use of larval extracts and/or purified antigens from both larval and adult worms would probably be more informative, providing information directly about which stage and/or antigen is responsible for inducing protective immunity to the parasite. The use of various strains of the mouse host would again help to explain

why some strains are easier to immunise than others.

Overall, it was seen that *H. polygyrus* provides a useful model for the study of a chronic nematode infection. The role of a number of immunological parameters in the control of the immune response to the parasite were studied in three strains of mice of varying response to infection. The stage specific expression of various enzymes and cuticular molecules was also investigated and it was noted that this correlated to the stage and immunogenicity of the parasite.

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