FACTORS INVOLVED IN IMMUNITY TO NEMATOSPIROIDES DUBIUS INFECTIONS IN MICE

by

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ABSTRACT

The present investigation examined the interaction between mouse peritoneal macrophages with various developmental forms of *Nematospiroides dubius* (*Heligmosomoides polygyrus*) a natural nematode parasite of the mouse and the ability of serum from mice immune to this infection to damage exsheathed L₃ in the absence of phagocytic cells.

Initial studies examined the adherence of peritoneal exudate cells to the surface of the parasite and the factors involved in promoting this adhesion. Peritoneal exudate cells from normal mice did not adhere to the cuticle of exsheathed L₃ in the absence of serum, but did so when the larvae had been sensitised with normal mouse serum. In this case the cells bound to the larvae via their C₃ receptors and confirmed previous observations that the parasite activated complement via the alternative pathway. It was also found that serum from mice immune to this infection contained antibodies directed against the cuticle of all larval stages as measured by the adhesion of cells to larvae that had been sensitised with heat inactivated serum. The cells in this instance adhered to the larvae via their Fc receptors. The factors involved in promoting adhesion of peritoneal exudate cells from normal mice and those from mice resistant to this infection were similar. The peritoneal exudate cells adhering to the larvae were found to be predominantly macrophages.

The cell adhesion studies demonstrated that all of the developing post-infective and the adult form of the parasite were capable of activating complement by the alternative pathway with the marked exception of larvae collected 96 hours post-infection. This
indicated a possible change in the antigenic structure of the cuticle.

Experiments were carried out to determine the ability of various immunoglobulin isotypes from mouse immune to reinfection to mediate cell binding to all developmental forms and adult worms of the parasite. It was found that the ability of IgM to promote the binding of cells to the larval stages increased dramatically from the pre-infective stages including sheathed and exsheathed L3 up to 96 hours post-infective larvae, but promoted little binding to adult worms. The IgG promoted cell binding in a similar pattern to that of IgM, but binding of the cells to 96 hours post-infective larvae was considerably reduced. Both IgG2a and IgG2b enhanced the binding of cells to pre-infective larvae, but were not active in promoting cell binding to post-infective larvae. However at a physiological level only IgG1 and IgM were active in promoting cell adhesion.

An in vitro assay to determine the effect of cell binding upon the infectivity of exsheathed L3 indicated that peritoneal exudate cells from mice infected with 2 doses of L3 were capable of damaging the larvae (as measured by a loss in infectivity) providing the larvae had been sensitised with antibody and complement or complement alone, but had no effect if the larvae had been sensitised with antibody alone even though antibody mediated cell adherence. Peritoneal exudate cells from normal mice were unable to damage the larvae even in the presence of antibody and complement.

Further in vitro studies showed that fresh untreated serum from mice infected with 4 doses of L3, IMS(4) was able to damage exsheathed L3 as measured by a reduction in their infectivity.
This effect was not apparent when fresh untreated serum from mice given 2 immunising doses, IMS(2) was used. The larvicidal activity was found only in long-term *N. dubius* infected mice since serum from mice infected with *Mesocestoides corti* an unrelated helminth parasite did not reduce the infectivity of the larvae. The larvicidal activity of IMS(4) appeared to be dependent on specific antibodies of the IgM class reacting with the cuticle of the larvae and binding complement.

Studies *in vivo* showed that fresh IMS(4) given intravenously to naive mice protected them against a subsequent intravenous challenge with exsheathed \( L_3 \), but not against a challenge with sheathed \( L_3 \). It was also found that serum from immune mice from which the greater proportion of the immunoglobulins had been removed was unable to transfer immunity passively to naive mice. The relative importance of these studies to other investigations dealing with nematode parasites is discussed.
STATEMENT

This thesis contains no material previously submitted by me for a degree in any university, and to the best of my knowledge and belief it contains no material previously published or written by another person, except where reference is made in the text.

Varunee Desakorn
December, 1983
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ABBREVIATIONS USED IN THIS THESIS

<table>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>c.a.</td>
<td>approximately</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis (β-aminoethyl) N,N'-tetraacetate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immuno-sorbent assay</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IMS</td>
<td>immune mouse serum</td>
</tr>
<tr>
<td>IMS(2)</td>
<td>serum from 2 times <em>N. dubius</em> infected mice</td>
</tr>
<tr>
<td>IMS(4)</td>
<td>serum from 4 times <em>N. dubius</em> infected mice</td>
</tr>
<tr>
<td>L₃</td>
<td>Third-stage (infective) <em>N. dubius</em> larvae</td>
</tr>
<tr>
<td>N.D.</td>
<td>not done</td>
</tr>
<tr>
<td>NMS</td>
<td>normal mouse serum</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>VBS</td>
<td>Ca⁺⁺, Mg⁺⁺ - supplemented veronal-buffered saline</td>
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CHAPTER 1

INTRODUCTION
1.1. GENERAL INTRODUCTION

Diseases caused by nematode parasites constitute a major health problem affecting a large proportion of the world's population particularly in tropical and subtropical countries. Although infections with many of these parasites are seldom fatal in man and animals they may lead to debilitating illness and there is little doubt that economic progress in some of the developing countries has been seriously impeded by such infections. A further problem that arises as a result of these infections is that the host's immune response to a variety of non-parasite antigens may be compromised. Thus in mice infected with *Ascaris suum* larvae, antibody formation against *Salmonella paratyphi* B was depressed. Likewise in children infected with ascarids there was a depressed antibody response to diphtheria and polioviruses and in adults a suppressed response to vaccines against typhoid and paratyphoid fever.

Recently a survey carried out by the World Health Organization (W.H.O. Technical Report, No. 666) has shown that in some countries more than 90% of the rural population may be infected with the hookworm *Ancylostoma duodenale* or *Necator americanus*. Infections caused by these parasites may give rise to a severe anemia (Gilles, Watson, Williams and Ball, 1964). *Ascaris lumbricoides* another nematode is one of the most prevalent of human metazoan parasites in the world today (Peters, 1978). Further the increase in new areas of land under permanent irrigation schemes has led to a continuous increase in the number of cases of schistosomiasis (W.H.O. Technical Report, No. 643).

One of the most effective ways to control some nematode infections such as hookworm would be to improve the sanitation and hygiene of the population in endemic areas. A simple provision such as the wearing of
shoes would in theory lower the rate of infection by the larvae of the hookworm since they infect their host by penetrating the skin usually of the feet. Further measures to prevent the use of night soil as a fertiliser would decrease the chances of infection. However, such apparently simple preventative measures for a variety of reasons are seldom achieved in even the more prosperous of the developing countries.

Current control of nematode infections by the use of chemotherapeutic agents or by using insecticides in an effort to control vectors such as those involved in the transmission of filariasis or onchocerciasis have been largely unsatisfactory. Whilst nematode infections particularly those where the adults live in the gut may be eliminated by the use of suitable antihelminthic drugs treated individuals rapidly become re-infected in endemic areas. Vector control through the use of insecticides has led to the emergence of resistant species and there is always the inherent danger of environmental pollution by the continued use or misuse of such agents. Thus in the latter case the cure may be more dangerous than the disease. A more satisfactory means of biological control lies in the development of suitable vaccines a goal that has met with limited success in the case of nematode infections.

1.2. VACCINATION OF HOSTS AGAINST NEMATODE INFECTIONS

Several methods of vaccination against nematode parasites have been employed, but with limited success. In the case of hookworm infection in dogs caused by *Ancylostoma caninum*, some resistance against further infection has been achieved by the subcutaneous injection of a vaccine containing the third-stage larvae of *A. caninum* which had been attenuated with x-irradiation or gamma radiation (Miller, 1971, 1975). Single and double vaccination schedules with these attenuated larvae,
were successful in protecting vaccinated dogs against the establishment of potentially severe challenge infections of normal larvae by both the subcutaneous and oral routes, and in preventing completely the morbidity and mortality associated with the challenge infection. Miller, (1965) showed that resistance to the challenge infection persisted for at least 7 months after completion of the vaccination schedule. An attenuated larval vaccine has also been used for immunising calves against Dictyocaulus viviparus. (Jarrett, Jennings, McIntyre, Mulligan and Urquhart, 1960). These investigators showed that calves developed a high degree of immunity to D. viviparus following the oral administration of third-stage D. viviparus larvae partially inactivated by x-irradiation. Recently there has been an attempt to control the parasite Trichinella spiralis by immunological means. The approach is based on the treatment of the parasite with a certain group of chemical agents related to the cyclohexanothioxanthones (Nabih, 1981). The chemically pretreated larvae were prepared by mixing the encysted larvae of T. spiralis isolated from the tongue muscles of infected mice by pepsin digestion with 1-ß-diethylamino-ethylamino-3,4,-cyclohexanothioxanthone. These treated larvae were centrifuged and suspended at a concentration of 500 larvae/ml in balanced salt solution. Mice were injected subcutaneously with two doses of 0.5 ml of the suspension of these chemically pretreated larvae, and were found to be protected against a subsequent oral challenge of normal living larvae. In addition, when two doses of these chemically pretreated larvae were given subcutaneously to mice already bearing a 6-week-old trichine infection the already established larvae were destroyed.

More recently the elucidation of the mechanisms by which animals develop a protective immune response to nematode parasites has received increasing attention due mainly to the fact that empirical immunising
procedures as used against bacterial and viral infections have been largely unsuccessful. (Terry, 1968). If such mechanisms were defined, attempts to stimulate protective immunity by vaccination could be made on a more logical basis. In recent years a great deal of work has been done on mechanisms of immunity to various nematode species and it would be pertinent at this stage to take some typical examples before discussing what is known regarding the mechanisms which render the mouse immune to *Nematospiroides dubius* infections.

1.3. MECHANISMS OF IMMUNITY TO *Nippostrongylus brasiliensis*

*Nippostrongylus brasiliensis* is a natural parasite of the rat which also has been adapted to infect the laboratory mouse (Wescott and Todd, 1966). Infections with this parasite have been used as a model to study both systemic and local immune responses as well as pathologic changes produced by nematode infections. The development of *N. brasiliensis* in the rat (Ogilvie and Jones, 1971) and mouse (Love, 1975) has been well characterized.

The adult forms of *N. brasiliensis* live in the lumen of the small intestine. Eggs are laid and pass out with the faeces and hatch into first stage larvae, which after two moult develop into infective third stage larvae (L₃). Once infective third stage larvae have penetrated the skin of the host, they are transported via the lymph and blood vascular systems to the lungs where they undergo a fourth moult. After a tracheal–oesophageal migration, the fourth-stage larvae (approximately 15-35% of the original L₃ dose) are carried to the lumen of the small intestine where they undergo a further moult and develop into adult worms. At 5-6 days post infection, egg laying commences. The adult population remains unchanged for about a further 5 days after
which time egg output drops quickly and the majority of the adults are rapidly expelled (Jarrett and Urquhart, 1971).

These workers considered the infection as being characterized by 4 phases:

1. **Loss phase 1.** The immobilization or destruction of larvae at the site of injection or on route to the intestine.

2. **Plateau phase.** The larvae which reach the small intestine develop to maturity and the numbers of adult worms remain static.

3. **Loss phase 2.** The majority of adult worms are rapidly expelled from the intestine in an exponential fashion.

4. **Threshold phase.** A small residual population of adult worms survive.

In animals undergoing multiple infections the loss phase 1 is increased with many larvae killed on route to the intestine.

Early studies on immunity to this infection, carried out by Sarles and Taliaferro, (1936) showed that in immune animals, some of the subcutaneously administered challenge dose remained trapped at the site of injection. More recently Love, Kelly and Dineen, (1974) showed that in immune rats, approximately 50% of the original challenge dose of L3 reaching the lungs, where delayed in there subsequent migration to the oesophagus. Furthermore, it was demonstrated that only a small percentage (17%) of the larvae reaching the oesophagus developed into adult worms, most of them being expelled from the small intestine before they matured (Love *et al.*, 1974; Love, 1975). The plateau phase was also shorter in these rats and the expulsion of adult worms from the small intestine was more rapid.

In 1936, Sarles and Taliaferro showed that protection against *N. brasiliensis* adult worms could be achieved in normal rats by passive
transfer of serum from hyperimmune animals. However, there was an enormous variation in the ability of different pools of antiserum to transfer protection and large volumes of antiserum were needed. Success in transferring immunity passively seems from a number of studies not to be related to the number of infections which the serum donors have undergone (Ogilvie and Jones, 1968; 1971; Wakelin, 1978). In contrast, Miller, (1980) has shown that the passive transfer of sera from rats immunized with two or three infections (hyperimmune sera) was more effective in determining worm expulsion than sera from rats recovered from a primary infection. The ability of the primary infection serum pools to protect animals on passive transfer against infection increased with time after infection but they were never as potent in their protective capacities as hyperimmune serum. The protection afforded by both primary infection serum and hyperimmune serum was dose-dependent, i.e., increasing protection was conferred with increasing doses of serum. There was also little variation in the ability of different pools of hyperimmune serum to cause expulsion. The consistently good protection carried out by Miller, (1980) when compared with the variable success of previous experiments (reviewed by Ogilvie et al., 1971) may be influenced by the strain and sex of the rats used (Dobson and Owen, 1978). The role of antibody as an essential component in mediating worm expulsion was further supported by the work of Sinski, (1981) who demonstrated that, when *N. brasiliensis* - infected rats were given cyclophosphamide as an immunosuppressive agent at the dose of 300 mg/kg, there was a significant delay in the expulsion of adult worms. The dose of cyclophosphamide used in his study had been shown previously to effect principally B-cell function (Turk and Poulter, 1972). Experiments by Jones, Edwards and Ogilvie, (1970) have shown that passive protection is conferred predominantly by an IgG fraction.
Previous work by Ogilvie, (1967) showed that rats infected with *N. brasiliensis* developed a striking increase in the level of serum IgE (reaginic or skin sensitising) specific antibody. This reaginic response has been shown to be a common feature of many helminth infections (Ogilvie and Jones, 1969; Sadun, 1972). Similar to other helminth infections, rats infected with *N. brasiliensis* not only show an increase in specific IgE antibodies directed against parasite antigens (Ogilvie, 1964; reviewed by Ogilvie et al., 1969), but also as a consequence of the infection IgE responses are potentiated against antigens unrelated to those of the parasite when simultaneously administered (Kojima and Ovary, 1975; Jarrett and Stewart, 1972; Orr, Riley and Doe, 1971).

Despite the fact that reaginic antibody is produced as a result of almost all helminth infections and the many studies into its possible role in protecting animals against infection, its true function remains unclear. It has been postulated for a number of these infections that upon contact between antigens and antibody of the IgE isotype, an anaphylactic reaction is elicited involving degranulation of mast cells and the release of vasoactive amines. It was proposed that because of these reactions the intestine becomes an unfavourable environment for the worm resulting in its expulsion from the host (Urquhart, Mulligan, Edie and Jennings, 1965; Mulligan, Urquhart, Jennings and Nielson, 1965; Panter, 1969b; Miller, 1971; Jarrett et al., 1971). In *N. brasiliensis* infected rats, the expulsion of the worms commences approximately 14 days after infestation (Ogilvie et al., 1971), whereas specific IgE antibody cannot be detected in the serum until between 16 and 21 days following infection (Ogilvie, 1967; Keller, 1970; Jarrett, Haig and Bazin, 1976; Allan and Mayrhofer, 1981). These antibodies are therefore detected
later than the commencement of worm expulsion, and the observation has contributed to the view that immediate hypersensitivity does not play a part in the expulsion of the worms (Ogilvie and Love, 1974). However, Allan and Mayrhofer, (1981) have studied the response of specific IgE antibody at the cellular level of infected rats by using the adoptive cutaneous anaphylaxis technique of Kind and Macedo-Sobrinho, (1973). They found that specific IgE antibody is secreted by the lymph nodes draining the lung and the gut at least 4 days before it can be detected in the circulation. Its absence in the serum at this time may be due to binding of IgE specific antibody to mast cells or macrophages (Capron, Dessaint, Joseph, Rousseaux, Capron and Bazin, 1977). These observations are consistent with other reports suggesting that systemic sensitisation of mast cells to parasite antigens occurs several days before specific IgE antibody can be detected in the serum (Urquhart et al., 1965; Wilson and Bloch, 1968; Church, 1975). The observations by Allan and Mayrhofer, (1981) suggests therefore that absence of circulating IgE in the serum of rats rejecting their worm burden does not constitute evidence against the participation of immediate hypersensitivity in the final phase of parasite expulsion. The involvement of the IgE isotype in immunity to *N. brasiliensis* is still unclear eventhough it is the only immunoglobulin isotype to increase in concentration during infection (Jarrett and Bazin, 1977). Indeed, Jones and co-workers, (1970) showed that fractions of antisera from rats, immune to *N. brasiliensis*, which were free of detectable IgE or IgG reaginic antibody, still had the ability to elicit the expulsion of adult worms. Furthermore, unsuccessful attempts were made to elicit the early expulsion of adult *N. brasiliensis* from the intestine of rats by the induction of severe anaphylactic shock using
adult worm antigens.

However, there is evidence that specific antibody can damage adult worms in vitro. Ogilvie and Hockley, (1968) demonstrated that antibodies cause severe changes to the cytoplasm of the gut cells of adult worms by noting their reduced ability to take up radio labelled orthophosphate from the host. Furthermore, Henney, Maclean and Mulligan, (1971); Lee, (1970; Edwards, Burt and Ogilvie, (1971); and Jones and Ogilvie, (1972) have shown that immune rats had specific antibody directed against the acetylcholinesterase produced by adult worms. The levels of acetylcholinesterase isoenzymes of worms from the plateau phase of infection differ from those of worms from the threshold phase. These antibodies may act by interfering with the function of the enzyme as a 'biological holdfast' forcing the worms to leave their position and migrate towards the gut lumen. The occurrence of lipid droplets and an accumulation of neutral lipid in antibody damaged worms supports the hypothesis that antibodies make the worms move into an environment where the oxygen potential is unfavourable to them (Lee, 1971). The fact that adult worms were not lost from irradiated rats following the administration of specific antiserum led Jones and Ogilvie, (1971) to suggest that a radiosensitive factor was required for rejection. They proposed that the mechanism of expulsion of adult worms occurs in 2 steps, firstly damage of adult worms by antibody which is then followed by a cellular dependent step,

Many experiments have shown that adoptive transfer of mesenteric lymph node cells from immune animals resulted in the rapid elimination of adult worms from normal and irradiated rats, suggesting that lymphocytes from immune animals may play a direct role in this process (Keller and Kiest, 1972; Kelly and Dineen, 1972; Dineen, Kelly and Love, 1973;
Kelly, Dineen and Love, 1973). Since mesenteric lymph nodes contain both thymus and bone marrow derived lymphocytes, adoptive transfer of these cells may confer on the recipient the ability to mount a delayed type and immediate type hypersensitivity response upon antigenic stimulation following infection. However, this situation was clarified by Ogilvie, Love, Jarra and Brown, (1977) who showed that immunity could be transferred to irradiated rats by thoracic duct lymphocytes from immune animals which had been depleted of Ig+ve cells. They showed also that the transfer of immune mesenteric lymph node cells did not consistently elicit the expulsion of adult worms from irradiated rats. This inconsistency might be explained by the fact that at times the mesenteric lymph nodes did not contain sufficient T cells to cause expulsion. This explanation was further supported by Grencis and Wakelin, (1982) who demonstrated that the cells from the mesenteric lymph nodes which appear to be involved in the mechanism responsible for worm expulsion were a population of rapidly dividing T lymphoblast cells and that these cells were present for only a limited period of time in the nodes after infection. Since the irradiated rats were not reconstituted with bone marrow and hence unable to produce antibody it seems possible that antibody was not directly involved in the rejection of the adult worms and that antibody dependent damage of adult worms was not a pre-requisite for expulsion. This is in agreement with the results of Jacobson, Reed and Manning, (1977) who showed that the rejection of adult worms still occurred in animals in which all immunoglobulin synthesis was suppressed by neonatal anti-\(\mu\) serum administration. Mitchell and co-workers, (1976) also showed that immune serum failed to expel adult worms in athymic mice, emphasising that expulsion is T-cell dependent. Furthermore, Nawa, Parish and Miller, (1978) have demonstrated that cells from the thoracic
duct lymph (T.D.L.) with no surface immunoglobulin, obtained from rats 10-11 days after a primary infection caused early elimination of both normal and 'damaged' worms. Nawa and Miller, (1978) showed also that the degree of expulsion was dependent upon the number of cells transferred. These results are inconclusive since T.D.L. have the capacity to adoptively transfer both humoral antibody and cell mediated responses. However, these results may lead to a re-evaluation of the 2-step theory of worm expulsion suggested by Ogilvie and Love, (1974).

Investigations have shown that infection with this parasite also stimulates a population expansion of intestinal mast cells which is found to be thymus dependent (Askenase, 1980; Burnet, 1977) and normally starts during the phase of immunological expulsion of the worms from the intestine (reviewed by Murray, 1972; Miller, 1981). It was proposed that infiltrating mast cells sensitised with specific IgE antibodies resulted in a local anaphylaxis following contact with parasite antigens resulting in elimination of the adult worms. However, the true immunological importance of the increase in number of mucosal mast cells is still unresolved. Studies carried out by Uber, Roth and Levy, (1980); Kojima, Kitamura and Takatsu, (1980); Crowle and Reed, (1981); Mitchell, Wescott and Perryman, (1983) indicated that mast cell-deficient w/w^V mice which had been infected with N. brasiliensis were able to expel adult worms as well as normal mice. A few mast cells were present in this strain of mouse in the intestinal mucosa and tongue, but their numbers did not change during the course of the primary infection. In contrast, worm expulsion in normal mice was associated with a moderate increase in numbers of intestinal mast cells, commencing at the onset of expulsion and peaking several days after expulsion was completed. This situation was further supported by experiments of other workers who
found that young or lactating rats (in which T-lymphocyte function is subnormal) do not expel adult worms when infected with N. brasiliensis despite normal parasite-specific antibody responses and normal numbers of functional mast cells (Jarrett, Urquhart and Douthwaite, 1969; Connan, 1973; Kelly et al., 1973). This suggests that another T-dependent cellular mechanism may be the critical effector in nematode expulsion.

Recently, Miller and Nawa, (1979) showed that there was an increase in the proportion of goblet cells in the jejunal mucosa and mucus secretion in rats infected with N. brasiliensis at the time of worm expulsion. Further evidence reported by Uber and co-workers, (1980) indicated also that intestinal goblet cell numbers and mucus secretion were increased during the course of N. brasiliensis infection in mast-cell deficient w/w^+ mice. These results uphold the hypothesis that goblet cells play a role in the expulsion of adult worms. The increased mucus secretion may act as a barrier in preventing the attachment of the parasite to the mucosa.

There is also some evidence suggesting that E-type prostaglandins (PG) may be involved in the expulsion of N. brasiliensis. Experiments have shown that the intraduodenal injection of chloroform extracts of acidified ram semen or of synthetic PGE_1 or PGE_2 into infected rats resulted in significant early worm expulsion (Dineen, Kelly, Goodrich and Smith, 1974a; Kelly, Dineen, Goodrich and Smith, 1974; Smith, Goodrich, Kelly and Dineen, 1974). It was also shown that the daily administration of inhibitors of PG biosynthesis prevented the expulsion of the parasite (Dineen et al., 1974a; Dineen, Kelly, Goodrich and Smith, 1974b). Dineen and Kelly, (1976) found that the level of prostaglandins of the "E" class increased 10-fold in intestinal tissues at the site of infection before the onset of worm rejection, and that E and F class prostaglandins both
caused contraction of isolated rat ileum (Bennett and Fleshler, 1970). Dineen et al., (1976) and Kelly and Dineen, (1976) reported that peak levels of PGE at the infection site in the gut were achieved on day 6 post-infection with *N. brasiliensis*. The raised levels of PGE may act on the worms indirectly by affecting gastrointestinal function which alters the micro-environment at the site of attachment. This may also be the reason for the increase in gut motility observed on day 8 after infection (Farmer, 1981). Furthermore, Richards, Bryant, Kelly, Windon and Dineen, (1977) demonstrated that exposure of worms *in vitro* to the action of PGE adversely affected their ability to re-establish in rats and caused structural alterations to their tissues. However, Kassai, Redl, Jecsai, Balla and Harangozo, (1980) have recently reported that they could find no substantial *in vitro* or *in vivo* untoward effect of prostaglandins on adult *N. brasiliensis* worms, and suggested that prostaglandins may not be directly involved in the immune rejection of *N. brasiliensis*.

In summary it appears that there is still a great deal of confusion concerning the mechanism of worm expulsion in this particular host-parasite relationship. What we may say with some certainty is that expulsion is an immunological phenomenon, appears to involve the participation of T cells but the relative role of specific antibody and cells in the process still remains obscure. What is perhaps also surprising is that little attention has been paid to the fate of infective larvae in immune rats during their migration to the small intestine. It is known that only a small proportion of the infective dose reaches the lumen of the small intestine and yet the factors involved in the arrest of their migration and the fate of the larvae remain largely unknown.
1.4. MECHANISMS OF IMMUNITY TO TRICHINELLA SPIRALIS

*Trichinella spiralis* is a gastrointestinal nematode infecting man, ruminants, pigs and rodents. This parasite develops through a series of enteral and parenteral stages. Infection occurs by ingesting meat containing muscle-stage larvae which are in the form of encysted larvae. The acidity of the stomach releases the larvae from the cysts which then enter the intestine and localize in the epithelial layer in the villus/crypt junction area of the small intestine (Gardiner, 1976) and moult twice through preadult to adult stages (Ali-Kahn, 1966; Kozek, 1971 a, b). After approximately 5 days post-infection, larvaposition begins and the newborn worms migrate through blood vessels, lymphatics or body cavities to striated muscle cells where they undergo a period of growth, differentiation and encapsulation to become muscle stage larvae (Ali-Kahn, 1966; Berntzen, 1965; Kozen, 1971 a, b). These encysted larvae may remain alive, despite calcification of the cysts, for a number of years. After copulation the males die and are expelled but the gravid female adult worms may survive for several weeks, depending upon the species of the host, and are finally expelled from the small intestine. Studies on immunity have been directed at the mechanisms involved in the expulsion of adult worms, and those leading to a reduction in the number of muscle stage larvae. It has been noted that most of the pathologic effects of infection arise from the passage of larvae through the body and their encystment.

Infection of most animals by *T. spiralis* results in high levels of IgE antibody (Ogilvie and Jones, 1969). Evidence reported by Dessein and co-workers, (1981) suggests that IgE antibodies may be critical in the development of resistance to this parasite. They showed that suppression of the total IgE antibody response in rats by
injection of rabbit anti-rat ε-chain antibodies during the course of a natural infection with *T. spiralis* resulted in a marked reduction in the number of eosinophils attracted to the *T. spiralis* larvae encysted in striated muscle. Blood eosinophilia following *T. spiralis* infection in IgE-suppressed rats was reduced in duration, appearing and terminating earlier than in control rats, but there was no consistent difference in the peak response in these animals. However, IgE-suppressed animals harbored two to three times more larvae encysted in their muscles compared with their control litter mates. It was proposed that, when the parasites invade the host tissues, IgE specific antibody in association with parasite antigens triggers the release of mediators from mast cells which mobilise eosinophils (Kay and Austen, 1971; Paterson, Wasserman, Said and Austen, 1976; Clark and Gallin, 1975; Goetzl and Austen, 1977), capable of damaging and killing the larvae prior to their encystment in smooth muscle (Dessein *et al.*, 1981).

In a natural infection with *T. spiralis*, an eosinophilia is induced within the first week postinfection, becomes maximal after 2-4 weeks depending on the host, and then tends to fluctuate over the ensuing months (Della Vida and Dyke, 1941, Basten, Boyer and Beeson, 1970). Previous studies in rats orally inoculated with *T. spiralis* indicated that development of the larvae to the preadult and/or adult stages was sufficient to elicit a peripheral blood eosinophilia (Zaiman and Villaverde, 1964; Lin and Olsen, 1974; Despommier, Weisbroth and Fass, 1974). In contrast, intravenous injection of newborn larvae did not stimulate an eosinophil response (Despommier *et al.*, 1974). These data suggested that the enteral (preadult-adult) stages are solely responsible for the eosinophilia during natural trichinosis. Data also indicate that the eosinophilia in rats and mice is primarily
mediated by thymus derived or dependent lymphocytes (Basten and Beeson, 1970; Basten et al., 1970). These data were supported by studies utilizing nude (Ruitenberg and Steerenberg, 1974) and thymectomized mice (Walls, Carter, Leuchars and Davies, 1973). In these mice there was a significant reduction in the blood and tissue eosinophil response following infection with *T. spiralis* compared with normal mice. The role of T lymphocytes in mediating the eosinophilia was further demonstrated by Bartelmez, Dodge and Bass, (1982), who showed that antigens prepared from all stages of the life cycle of this parasite are capable of inducing non-adherent spleen cells recovered from animals infected with *T. spiralis* to release an eosinophil growth factor into the culture medium upon incubation in vitro. When eosinophils in mice infected with *T. spiralis* were depleted by injections of a rabbit anti-mouse eosinophil serum, the rate of expulsion of adult worms was not affected but there was a doubling in the number of muscle-stage larvae, compared with control animals (Grove, Mahmoud and Warren, 1977), suggesting that the eosinophils may play a crucial role in resistance to the larvae but not to the adult stage. This hypothesis was supported by Kazura and Aikawa, (1980) and Kazura, (1981) who showed that eosinophils, in the presence of serum from *T. spiralis* infected animals, destroyed the newborn larval stage of the parasite in vitro. This eosinophil-mediated cytotoxic effect was dependent on IgG antibodies which appeared in the serum 3 weeks after a *T. spiralis* infection and which reacted only with the newborn larval stage of the parasite. Further, the killing of newborn larvae was independent of complement (Kazura, Aikawa and Grove, 1978).

Immunity to *T. spiralis* as expressed by accelerated expulsion of the adult worms may be transferred to normal rats with serum from previously infected immune rats. The degree of protection was related
to the number of doses of immune serum that was transferred (Love, Ogilvie and McLaren, 1976). In contrast, other workers have failed to transfer immunity with a specific antiserum, but have used relatively small amounts of serum (Crum, Despommier and McGregor, 1977).

An indication that antibody was involved in expulsion of the adult worms was supported by the transfer of immunity to rats by a purified B lymphocyte population administered intravenously 2 days prior to oral challenge with muscle larvae. Ten days after transfer there was an enhanced loss of adult worms which greatly reduced the numbers of larvae encysting in the muscles (Despommier, Campbell and Blair, 1977). Since immunity could also be transferred with a sensitised population of thymus derived lymphocytes the antibody involved probably results from the interaction of these lymphocytes and host B lymphocytes (T dependent antibody). Despommier and co-workers, (1977) also transferred to normal rats thoracic duct lymphocytes from rats, which had been immunized with the early intestinal phase of infection, and found that these rats eliminated adult worms more rapidly than did the control animals. They found that these same cells were ineffective if the rats were challenged intravenously with newborn larvae as evidenced by similar numbers of muscle larvae recovered by whole carcass digestion from the adoptively-immunised animals and their respective controls 30 days later. These results suggested that, although both the intestinal and parenteral phases of the infection stimulate good immunity, the protection seems to be stage-specific (James and Denham, 1975; James, Moloney and Denham, 1977). This hypothesis was further supported by Moloney and Denham, (1979) who showed that transfer of serum from mice immunised with either newborn larvae or infective larvae to normal mice always gave good protection upon subsequent challenge of these mice with newborn larvae as evident
by a reduction in the number of muscle larvae. If newborn larvae were incubated in serum from immunised mice their infectivity was reduced, but if the serum was pre-adsorbed with newborn larvae this effect was lost. Such adsorbed serum, did not confer immunity on recipient mice to this infection. In addition, spleen cells from mice immunised with newborn larvae when transferred to recipient mice protected them against a subsequent challenge with newborn larvae as measured by a reduction in the number of muscle larvae, whereas those from mice immunised with infective larvae did not. Immunity to *T. spiralis* as measured by accelerated worm expulsion may also be transferred adoptively with mesenteric node lymphocytes (Wakelin and Wilson, 1977). The cells capable of transferring immunity were present in the mesenteric lymph node for only a limited period of time after infection and found to be predominantly Ig-ve and presumed to be T cells (Grencis and Wakelin, 1982; Wakelin, Grencis and Donachie, 1982; Wakelin and Wilson, 1979). It has been proposed that a major function of T cells in the expulsion of *T. spiralis* is the mediation of inflammatory changes in the intestine, in co-operation with myeloid cells of bone marrow origin (Wakelin et al., 1979). Although the details of this mechanism are unknown, it has been shown that mesenteric lymph node cells taken at times when T lymphoblast activity is high will transfer an enhanced mucosal mast cell response (Alizadeh and Wakelin, 1980). It therefore seems that it is the lymphoblast population or its immediate progeny which initiates the sequence of intestinal inflammatory changes that lead ultimately to worm expulsion.

In 1975, Larsh and Race proposed a two-step mechanism for the expulsion of adult *T. spiralis* from the host gut. The first step is an immunologically specific delayed hypersensitivity reaction between antigen-sensitive T cells and *T. spiralis* antigen which results in
injury to host gut tissues. This is followed by a nonspecific inflammatory reaction to injury caused during the first step and leads to changes in the enteromicroenvironment which is unfavorable for the continued existence of adult worms. Other workers have shown that nonspecific inflammation induced by previous infections with *Nippostrongylus muris* (Louch, 1962), *Ancylostoma caninum* (Cox, 1952; Goulson, 1958), *Salmonella typhimurium* (Brewer, 1955) and *Eimeria nieschulzi* (Stewart, Reddington and Hamilton, 1980), results in dramatic alterations in the enteric environment which adversely affect the establishment of *T. spiralis* leading to the rapid expulsion of the adults. Alizadeh and Wakelin, (1982) showed that challenge infections with infective larvae given shortly after expulsion of a primary infection (day 14) were expelled rapidly, worm loss being virtually complete within 24 hours. They suggested that either the specific inflammatory changes generated during primary infection resulted in an environment that was unsuitable for establishment of subsequent infections or that challenge infections provide a stimulus that can provoke an almost instantaneous response in the primed intestine. In mice this response to challenge was short-lived and persisted only until day 16 after the primary infection. After this time, challenge worms were expelled more slowly. However, in rats the rapid expulsion response was expressed for at least 7 weeks after a primary infection. These species differences in ability to respond rapidly to challenge is also supported by the work of Bell and McGregor, (1980) who showed that rapid expulsion is genetically determined.

Infections with *T. spiralis* induce an inflammatory reaction in the tissues of infected animals, which involves a variety of leucocytes such as neutrophils, eosinophils, mast cells, basophils and mononuclear cells. Eosinophils and macrophages have been shown to rosette together in the
peritoneal cavity of rats after a second intraperitoneal injection of infective muscle larvae (Walls, Hersey and Quie, 1974). Eosinophils were closely associated with macrophages rather than with other cell types or with parasites. A striking collection of eosinophils around individual macrophages was noted 48 hours following the second challenge. Immunofluorescent studies suggested that the eosinophil-macrophage rosettes were mediated by the presence of immunoglobulin on the surface of the macrophages. Additional evidence for the role of immunoglobulin resulted from observations in vitro where it was found that the numbers of rosettes were reduced following prior incubation of the macrophages with trypsin, which presumably removed cytophilic antibody (Nelson and Boyden, 1967).

Mackenzie and co-workers, (1981) studied the adherence in vitro of various cell types that occur in the inflammatory response to the surface of various stages of T. spiralis in the presence of serum from normal or T. spiralis infected rats. Adherence of the cells to the parasites occurred either via Fc receptors or via complement-binding sites. However, differences not only in the way these various cells adhere to the parasites were observed but also differences in the length of time they remain attached to the parasites. Mast cells adhered for a short time, did not flatten onto the surface and did not degranulate, adherence ceased after 4-6 hours. Eosinophils adhered within minutes to the surface of worms, flattened and degranulated, only cytoplasmic remnants being seen after 24 hours in culture. In contrast, only a small area of the cytoplasmic membrane of neutrophils flattened onto the surface of the worms. The neutrophils detached within 2-24 hours leaving "footprints" on the surface of the parasite. Macrophages adhered permanently to the surface of worms, did not flatten and retained their integrity. When macrophages and eosinophils were present together the eosinophils adhered
first and degranulated. The macrophages interacted with the surface of the parasite later and phagocytosed the eosinophil debris. Mackenzie and co-worker; (1980) also demonstrated that the cuticle of infective larvae and adult worms of T. spiralis activated complement via the alternative pathway, but the cuticle of newborn larvae did not, which indicated that the cuticle of newborn T. spiralis larvae was different from that of either the infective larvae or adult worms. However, when newborn larvae were grown in media overnight and then exposed to fresh normal serum, a narrow band in the mid region of some larvae activated complement as measured either by cell adherence or by using fluorescently labelled specific antibody to C3. In vitro studies also showed that the infective and newborn larvae of T. spiralis were killed by eosinophil-enriched cell populations in the presence of specific antibody as evidenced by the larvae bursting and extruding their internal organs through the cuticle (Mackenzie, Preston and Ogilvie, 1978; Kazura and Grove, 1978; Mackenzie et al., 1980; Kazura et al., 1980). The speed of killing of the larvae by eosinophils was enhanced when the immune serum was freshly collected and when the eosinophil suspension also contained neutral red-positive nonadherent macrophages (Mackenzie et al., 1980).

Philipp and co-workers, (1981) demonstrate that in a primary infection in rats with T. spiralis, serum obtained one week after infection contained antibodies to surface antigens of both infective larvae and intestinal worms as shown by an immunoprecipitation assay. These early sera, however, failed to react with new born larval surface antigens, indicating that there are none or few shared antigenic determinants between the surface proteins of newborn larvae and the other two stages. From day 12 onwards after the infection, the concentration of antibody to surface antigens of infective larvae increased whereas antibody to adult
worms decreased as measured by an immunoprecipitation assay. An absence of cross-reactivity can, however, be concluded from the finding that adsorption of immune rat sera with intestinal worms removed antibodies to their surface antigens as expected, but not to the surface antigens of infective larvae as measured by an immunoprecipitation assay. Conversely, adsorption of sera with infective larvae removed only antibodies to the surface antigens of this stage. In addition, the time course of appearance of antibodies to the surface antigens paralleled the appearance of antibodies that mediate eosinophil adherence to the surface of each stage of the parasite. From these results it was concluded that the T. spiralis displays stage specific antigens and these could be targets for the antibody-dependent destruction of this parasite, by eosinophils known to occur in vitro.

1.5. **NEMATOSPIROIDES DUBIUS A NATURAL NEMATODE PARASITE OF THE MOUSE**

1.5.1. **LIFE CYCLE**

Nematospiroides dubius ( = Heligmosomoides polygyrus), a trichostrongyloid nematode parasite of mice, was first described by Baylis, (1926) when he isolated it from the woodmouse (Apodemus sylvaticus). The life cycle has been studied in detail by a number of investigators including Spurlock, (1943); Baker, (1954); Ehrenford, (1954); Fahmy, (1956); Dobson, (1960); and more recently by Bryant, (1973) who has comprehensively reviewed the earlier data.

The eggs, (70-84 microns long by 37-53 microns wide) passed in the faeces of infected animals contain fully developed larvae within 24 hours after being laid. The eggs hatch after 36-37 hours to liberate the first larval stage (L1), which moult 28-29 hours later to give rise to
the second larval stage (L₂). At approximately 4-5 days of age, the larvae undergo a partial moult to become ensheathed, non-feeding but active, infective third stage larvae (L₃). These infective third stage larvae, when ingested by the host, exsheath in the stomach within 5 minutes (Sommerville and Bailey, 1973). The exsheathed larvae then pass into the small intestine and by 24 hours, (Bryant, 1973), most of them have penetrated to the muscularis mucosa. At 48 hours post infection differentiation to male and female worms was apparent. The larvae moult once more, 90-96 hours after penetrating the intestinal wall to become fourth stage larvae. A possible further moult took place at 144-166 hours post infection and by 191 hours most of the worms had passed from the mucosa into the intestinal lumen to take up their adult position attaching themselves to the wall of the small intestine by intertwining extensively with the gut villi (Kleinschuster, Hepler and Voth, 1978). The first eggs may be detected in the host faeces 240 hours after infection.

1.5.2. PATHOLOGY OF THE INFECTION

A heavy infection of N. dubius in mice causes progressive emaciation, sensitivity to cold, roughness of the coat and reddish-brown diarrhoea, probably due to either severe inflammation of the intestinal mucosa or intestinal petechial haemorrhages (Spurlock, 1943). These haemorrhages caused by the larvae penetrating the wall of the small intestine were thought to be due to the release of necrotising substances (Baker, 1955; Liu, 1965a). Baker, (1954) also mentioned that possibly secretory products from the larvae destroy the glandular epithelium. As a consequence of this, the small intestine becomes inflamed, enlarged and fragile. Furthermore, lymphadenitis and hyperplasia of the reticuloendothelial tissue in the mesenteric lymph glands. Nonspecific hepatitis and splenomegaly were also found and assumed to be due to
the mechanically produced tissue damage resulting from larval invasion of the intestine and to the allergenic nature of the substances released by the parasites (Liu, 1965a).

Four hours following ingestion, penetration of the intestinal epithelium commenced and after 12-24 hours the basement membrane had ruptured and necrosis in the muscularis mucosa was noticed (Liu, 1965a). The necrotic areas were intimately surrounded by a neutrophilic infiltration (Liu, 1965a; Panter, 1969a). By the 2nd day after infection, haemorrhages in the small intestine were visible and increased both in number and size until the 4th day. By this time, the larvae began to encyst in the muscularis mucosa and macrophage infiltration occurred (Baker, 1954; Jones and Rubin, 1974). By the 6th day, the cysts were white, opaque and purulent, and the larvae began to emerge from the cysts as juvenile worms. The vacated cysts then became filled with cells, mainly neutrophils and macrophages and a few eosinophils (Baker, 1954; Liu, 1965a; Panter, 1969a; Jones et al., 1974). The nodules increased in size until about the 13th day, after which they regressed and the lesions were repaired completely within 21 days after larval ingestion (Liu, 1965a; Baker, 1954). The adults appeared in the lumen, deeply entwined around the villi with their anterior end deep in the Crypts of Lieberkuhn. There was marked atrophy of the mucosa (Baker, 1954) and Panter, (1969a) claimed that the worms fed on host tissue.

Upon subsequent re-infection, after 2 days there was marked necrosis of the mucosa and a cellular infiltration by polymorphonuclear cells, plasma cells macrophages and eosinophils (Liu, 1965b). The cysts were surrounded by an eosinophilic halo and the emergence of juvenile worms was greatly delayed (Jones et al., 1974). The lesion then developed a necrotic centre and appeared as yellow or white granulomata. Forty nine days after larval ingestion fibroblasts were
present in the lesions and by day 73 regression had commenced, so that by day 90 few granulomata remained.

1.5.3. **INDUCTION OF IMMUNITY IN MICE TO INFECTION WITH**

**N. DUBIUS**

It is clear from the literature that following a primary infection with *N. dubius* mice develop a degree of resistance to a subsequent infection. This resistance to re-infection is related not only to the number of previous infections but also to the strain of mouse (Van Zandt, 1961; Panter, 1969b; Bartlett and Ball, 1972; Chaicumpa, Prowse, Ey and Jenkin, 1977a; Prowse, Mitchell, Ey and Jenkin, 1979a). What is also apparent from the literature is that the immunity appears to be induced only by the living parasites and not by their excretory/secretory products given alone or by larvae killed by freezing and thawing. Furthermore adult worms implanted into the small intestine of naive mice do not themselves induce a state of resistance (Jacobson, Brooks and Cypess, 1982). In contrast, Hurley, Day and Mitchell, (1980) have shown recently that small numbers of adult worms injected intraperitoneally are capable of inducing accelerated rejection of worms arising from a subsequent oral 

The apparent inability to induce immunity by other than by using living parasites is not a phenomenon confined only to this particular host/parasite relationship. Induction of immunity to many intracellular parasites such as bacteria may be brought about only by using living vaccines. The reason for this is that for good immunity one requires two effector mechanisms, an 'activated' cellular response as well as the production of humoral antibody. For reasons still unclear killed vaccines in general fail to 'activate' the cellular effector arm.
It will be pertinent now to discuss what is known regarding the development of immunity to *N. dubius* infection in mice and the changes in the host's immune system that take place during this development.

The development of resistance in mice to an infection by *N. dubius* was first described by Van Zandt, (1961) who found that mice given three oral infections of *L* displayed some resistance to further re-infection as measured by the number of adult worms in the intestine of the animals compared with the number recovered in the controls. More recently, other workers have shown that the degree of resistance is dependent on the number of living larvae used in the immunising dose and/or the number of doses given (Panter, 1967; 1969b; Bartlett et al., 1972; Hosier and Feller, 1973; Chaicumpa et al., 1977a). Furthermore, Leuker, Rubin and Anderson, (1968) found that immunity in mice may be effectively induced by injecting them subcutaneously with 4,000 exsheathed larvae. This was confirmed by Rubin, Leuker, Flom and Anderson, (1971) and Cypress and Zidian, (1975). A limited number (0.005%) of the vaccine dose was recovered in the intestine as adult worms.

Van Zandt, (1961) and Panter, (1969b) suggested that immunity was initiated by the *L* and not by the adult worm. This suggestion was confirmed by Bartlett et al., (1972) and by Chaicumpa et al., (1977a). Panter, (1969b) suggested also that immunity was due to an immediate type hypersensitivity reaction. She demonstrated that multiply infected mice mounted active cutaneous anaphylactic (A.C.A.) responses to extracts of adult worms, and sera from these mice could be used to induce passive cutaneous anaphylactic reactions (P.C.A.) with the adult worm antigen extract. She showed that immune mice were also subject to anaphylactic shock on intravenous injection of worm antigens together with Evans blue. The intestines of such mice showed an obvious
distension and blueing of the parasitized part of the small intestine. It was also found that *N. dubius* infected mice sensitised to horse serum rejected their adult worms when challenged intra-peritoneally with horse serum. It was suggested that in immune mice a fresh intake of larvae initiated an anaphylactic reaction, and that changes associated with this reaction prevented a large proportion of the inciting larvae from becoming established. More recently, Jones *et al.*, (1974) showed that most of the larvae in the challenge infection presented to mice that had been given previously 3 doses of 200 L₃ orally were rapidly expelled from the host prior to their invasion and maturation within the muscularis mucosa as evidenced by fewer larvae and active granulomatous lesions in the small intestine. They suggested that an immediate hypersensitivity response may have largely pre-empted the needed participation of cellular mechanisms of immunity in these mice which supported the results of Panter, (1969b). In contrast, the number of adult worms arising from a challenge infection in mice that had been given previously 2 doses of 2,000 exsheathed L₃ subcutaneously were much greater. In addition, larval development was said to proceed more normally in subcutaneously sensitised mice than in orally sensitised mice.

Jones *et al.*, (1974) suggested with no supporting evidence that in orally sensitised mice the challenge larvae were expelled from the small intestine prior to penetration whereas in subcutaneously sensitised mice the larvae were killed following penetration of the muscularis mucosa. This concept was further supported by Jones, (1974) who showed a greater active cutaneous anaphylaxis reaction in orally sensitised animals when compared to subcutaneously sensitised animals. This was determined by comparing the mean diameter and the intensity of the reactions of skin lesions after 30 minutes following the intradermal
injection of larval or adult antigen and intravenous injection of Evans blue. From this it was concluded that there was a higher production of reaginic antibody following oral immunisation than after subcutaneous immunisation, indicating that orally immunised mice had a greater potential than subcutaneously immunised mice for immediate hypersensitivity reactions. When subcutaneously immunised mice were given anti-thymocyte serum and cyproheptidine as immunosuppressive agents the immunity was abrogated but not in orally immunised animals. It was suggested that the removal of challenge larvae from the muscularis mucosa of subcutaneously sensitised mice involved sensitised lymphocytes, eosinophils, antiworm antibody and histamine, whereas the immune expulsion in orally immunised mice was due to an immediate hypersensitivity reaction. This evidence indicated that the mechanisms of resistance to infection displayed by immunised mice depended on the route used for immunisation. However, these ideas were opposed by Chaicumpa et al., (1977a) who showed that mice may be effectively immunised with living L₃ using a variety of injection routes including oral, intravenous, intraperitoneal and subcutaneous. The immune mechanisms involved in the expression of resistance operated on the larvae after they had penetrated the wall of the small intestine irrespective of the route used for immunisation. This was determined by making use of the formation of granulomas around a foreign body. The investigators argued that if the larvae were expelled in immunised mice prior to penetration then the numbers of granulomas seen in the challenged immune mice should be no different from that observed in unchallenged immune mice, the number of granulomas seen here being due to the immunising dose(s). On the other hand if the larvae penetrated the wall of the intestine then the number of granulomas should be increased in the challenged group of mice by an amount bearing
some relationship to the challenge dose. The latter proved to be the case and it is now generally accepted that immune mice do not expel a challenge dose of larvae from the lumen of the small intestine, but the larvae are killed in the muscularis mucosa.

Cypess et al., (1975) found that mice immunised by repeated oral infection lost their adult worm burden resulting from the immunising doses following challenge with L₃. The expulsion of adult worms appeared to parallel the self cure reaction first observed by Stoll, (1929) in his work with lambs infected with Haemonchus contortus. The observation was supported by Cypess and Van Zandt, (1973) who showed that following the ingestion of L₃, mice that had been subjected to three previous infections rejected the resident adult worms arising as a result of the previous infections within 4 hours. Recent investigations by Prowse et al., (1979a) in seven different inbred strain of mice showed that adult worms arising from previous infections were eliminated albeit slowly in certain strains but not in others on subsequent oral challenge. They did not observe the rapid expulsion described by Cypess et al., (1973). The ability of immune mice of certain strains to reject their worm burden appeared to be T cell dependent since the adults were not lost from athymic (nude) mice. However there was some elimination if these mice were reconstituted with T cells (Prowse, Mitchell, Ey and Jenkin, 1978a).

1.5.4. CHANGES IN IMMUNOGLOBULIN LEVELS DURING INFECTION

Crandall, Crandall and Franco, (1974) found that serum IgG₁ was considerably increased as measured by radial immunodiffusion following a primary infection of mice with N. dubius. Specific antibody in this immunoglobulin class was detected within the first 2 weeks of infection by an indirect fluorescent antibody technique using cryostat sections of adult worm as antigen. More recently, Day, Howard, Prowse, Chapman
and Mitchell, (1979) found that heat-labile circulating reagins which were specific for ES (Excretory/Secretory) products appeared in the serum of mice 20 days post infection. These reagins were shown to be characteristic of rat skin-fixing reagins (IgE antibodies) as assayed by passive cutaneous anaphylaxis (PCA). Crandall et al., (1974) found that there was also an increase in the levels of IgM, IgA and IgG₂ but to a much lesser extent than the increase in IgG₁.

Studies on the intestinal cellular responses after challenge of singly-infected mice with larvae, showed by immunofluorescence that IgG₁ plasma cells were the most common infiltrating cell. In intestinal washes, IgA and IgG₁ were detected, but specific antibody was detected only in the IgG₁ isotype which is homocytotropic in the mouse (Crandall et al., 1974). However, Cypress, Ebersole and Molinari, (1977) found that antibody in the IgA class could also be detected in intestinal washings. They used the antibody-binding technique previously described by Nash and Heremans, (1969). The technique was based upon a determination of the level of IgA before and after removal of specific antibody from the sample. Removal of anti-N. dubius antibodies, both precipitating and nonprecipitating, was accomplished by reacting the intestinal washings with an excess of crude homogenized adult N. dubius and then removing the complexes by precipitation with rabbit anti-adult worm antiserum. It was not known whether the majority of IgA antibodies detected in the intestinal washings were of local origin. No IgM or IgG₂ antibodies were detected in the intestinal washes. They suggested that the increase in IgG₁ in the intestinal washings was due to leakage of serum proteins into the lumen associated with the time of larval emergence and/or the establishment of the adult population. The levels of IgG₁ and IgA in serum were increased and antibodies to crude extract adult antigen in both immunoglobulin classes were detected
After multiple infections, there was an extraordinary increase in IgG$_1$ as high as 35-50 mg/ml and antibody in this isotype was always detected (Crandall et al., 1974; Molinari et al., 1978; Prowse, Ey and Jenkin, 1978b). The unusually high IgG$_1$ level has been observed in a number of chronic infections caused by metazoan parasites (Mitchell, Marchalonis, Smith, Nicholas and Warner, 1977a; Mitchell, Goding and Rickard, 1977b; Sher, McIntyre and Lichtenberg, 1977), and may reflect chronic exposure to large amounts of antigens. The immunological importance of this amazing increase in the level of IgG$_1$ is still unclear although it has been suggested that it may be parasite protective rather than host protective (Mitchell et al., 1977a). The reason for this suggestion was due to the fact that IgG$_1$ was supposed to be a non complement fixing immunoglobulin. Recent studies by Ey, Russell-Jones and Jenkin, (1980) indicate that this supposition was not justified and the idea that IgG$_1$ is parasite protective needs re-evaluating.

1.5.5. CHANGES IN CELL LEVELS DURING INFECTION

It has been shown that following a single oral or intravenous infection there was a five-fold increase in the total blood leucocyte count. The cell levels peaked 6-8 days after the infection when the emerging juvenile worms were migrating back into the lumen of the small intestine, then returned to normal levels by day 11-12 (Baker, 1962; Prowse et al., 1978b). In contrast, the increase in the number of peritoneal exudate cells was still elevated at this time. The initial changes in the blood leucocyte count seemed to be due mainly to an increase in the numbers of lymphocytes and neutrophils. However, in the peritoneal cavity both macrophages as well as lymphocytes and neutrophils increased in number. Although the relative increase of lymphocytes and neutrophils in the peritoneal cavity
exceeded that of macrophages (>20 fold compared to 2 to 4 fold), the absolute number of macrophages was always three to four times that of the other two cell types. Baker, (1962) demonstrated that the leukocytosis was not due to a bacterial infection or anaemia resulting from larvae penetrating the wall of the intestine. More recently, Cypess, (1974) found that in reinfected animals there was a reversal of the blood neutrophil/lymphocytes ratio which he considered to be due to inflammation about the time of larval killing. However, the most dramatic change seen following a secondary infection was in the number of circulating eosinophils. These cells were virtually absent from the blood and peritoneal cavity until day 7 after the 2nd infection when they appeared suddenly and were maintained for a number of weeks (Prowse et al., 1973b). This indicated that the eosinophilia was absolutely dependent on a secondary stimulation. Their appearance appeared to correlate with an increase in immunity to further challenge suggesting that eosinophils may play an important role as an effector cell in immunity to this parasite. At present, it is not clear whether in vivo the eosinophils are actively involved in the killing process or attracted to the site and play a secondary role once the larvae are killed. An eosinophilia is characteristic of many metazoan infections and although they appear to kill some parasites in vitro under the appropriate conditions such as schistosomulae, their function in vivo is still unclear (Archer, 1963; Beeson, 1977; Zucker-Franklin, 1974).

1.5.6. **THE ROLE OF PHAGOCYTIC CELLS AND ANTIBODY IN IMMUNITY TO N. DUBIUS INFECTION**

It is clear from the literature that mice given living \( L_3 \) develop resistance to further infections. The degree of immunity is related to the number of infections that the mice have been exposed to and it appears
that mice of different strains may not develop similar levels of resistance (Spurlock, 1943; Cypess et al., 1975; Leuker and Heppler, 1975; Prowse et al., 1979a). There is also a strong T cell component in the development of this resistance (Prowse et al., 1978a) and during its course, as has been discussed above, there are marked changes in the levels of certain cells in particular eosinophils in the blood and peritoneal cavity as well as a dramatic increase in the serum concentration of IgG₁. In general with one exception (Behnke and Parish, 1979) investigators have failed to transfer immunity passively to naive mice with serum from immune mice (Panter, 1967, 1969b; Cypess, 1970; Chaicumpa, Jenkin and Rowley, 1976). The one exception occurred in NIH mice. However it is clear from the in vitro work of Chaicumpa and Jenkin, (1978) that specific antibody may play an important role in vivo in mediating immunity although one requires in addition changes in the activity of the phagocytic cells. They found that peritoneal exudate cells in the presence of immune serum were able to damage L₃ as measured by a loss of infectivity. In contrast cells obtained from normal mice were inactive in this respect under the same experimental conditions even though they adhered strongly to the cuticle. More than 90% of the cells adhering to the larvae were macrophages and histochemical studies on the cells from immune mice indicated that the macrophages were in an activated state. The ability of Behnke and Parish, (1979) to transfer immunity passively with serum to NIH mice might be related to the fact that these mice may possess macrophages in an already activated state as has been reported for other strains of mice. The NIH strain of mice are known to respond more rapidly to several intestinal nematodes (Wakelin, 1975; Wakelin and Lloyd, 1976). It is also interesting to note that the effects of the immune serum were not observed if administered after day 6 of infection, which again suggests that the immune mechanisms operate primarily on the
larvae during their early development in the muscularis mucosa. Further work by Williams and Behnke, (1983) supported these initial observations although in this investigation the infective larvae were exposed to 5 k rads of cobalt 60 irradiation since Behnke and Parish, (1981) had shown that irradiated larvae are particularly sensitive to the immune responses of the host. Recently again using irradiated infective larvae, Pritchard, Williams, Behnke and Lee, (1983) have shown that the protective effect of the immune serum resided in the purified IgG1 fraction which also promoted the adherence of peritoneal exudate cells to the worm surface. However these in vivo studies although important are not helpful in understanding further the mechanism through which immune serum operates in promoting resistance. Recent studies both in vitro and in vivo have shown that the effector cells involved in immunity may not only be macrophages but also neutrophils and eosinophils (Penttila personal communication).

Increased knowledge of the mechanisms of immunity to N. dubius infection in mice may help in our understanding of the complex interplay between host and nematode parasites of medical importance.

The present thesis examines in detail the reaction of mouse peritoneal macrophages with infective L3 and the ability of immune serum to damage the larvae in the absence of phagocytic cells.
CHAPTER 2

MATERIALS & METHODS
2.1. INCUBATION MEDIA AND DILUENTS

2.1.1. INCUBATION MEDIA

The basic medium used was 199 Medium (Flow Laboratories, U.S.A.) containing penicillin and streptomycin sulphate (each 200 units/ml) and 5-fluorocytosine (10 µg/ml). In the preparation of parasites, the basic medium was buffered with NaOH + N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; Calbiochem; 10 mM final concentration) to pH 7.4, termed medium A. Supplemented medium A contained 10% heat-inactivated foetal calf serum. For cell-parasite cultures, the basic medium was buffered with NaHCO₃+HEPES (10 mM) to pH 7.4 and supplemented with heat-inactivated foetal calf serum (5%), termed medium B. In harvesting peritoneal exudate cells, 5 units heparin/ml of medium was added to medium B to make heparin-medium B.

2.1.2. DILUENTS

The diluent used for cell adherence assays and haemolytic complement assays was Ca⁺⁺, Mg⁺⁺ - supplemented veronal-buffered saline (VBS : 5mM Sodium barbitone, pH 7.4/0.142M NaCl/0.15mM CaCl₂/0.5mM MgCl₂). As indicated in the text, this diluent contained in addition 10mM EGTA (VBS-EGTA) or 10mM EDTA (VBS-EDTA). PBS was 13mM Sodium phosphate-buffered saline, pH 7.4.

2.2. MICE

All mice used in this study were outbred LACA mice raised under specific pathogen free, (S.P.F.) conditions in a closed colony at the Central Animal House, University of Adelaide. They were transferred to conventional conditions at the age of 6 weeks and used 2-3 weeks later.
2.3. MAINTENANCE OF THE PARASITE NEMATOSPIROIDES DUBIUS (HELIGMOSOMOIDES POLYGRUS).

Third-stage infective larvae (L₃) of *N. dubius* were supplied originally by Dr. R.I. Sommerville, Zoology Department, University of Adelaide. The parasite was maintained by infecting male or female mice orally with 200-400 L₃. These infected mice were used as a source of eggs. Fresh mice were infected on average, every 3-4 months. The faeces of the mice were collected on the 9th day after infection by placing wire mesh grids in the bottom of the cage lined with moist filter paper. The faecal pellets were mixed with distilled water and after standing at room temperature for 15 minutes, were passed through a fine 500μ mesh. After centrifuging the filtrate at 1500g for 15 minutes, the supernatant was discarded and the deposit resuspended in distilled water. The suspended sediment was then streaked onto moist Whatman filter paper lining a large petri-dish or a 30x30 cm glass tray. These were then left at room temperature under high humidity (Fig. 2.1.).

After seven days of culture the larvae were collected by rinsing the filter paper in the petri-dish with distilled water. By this time the larvae were in the third stage, which is the infective stage. They were washed and re-suspended in distilled water and kept at 4°C until required. The L₃ could be maintained at this temperature for periods of up to three months and remained fully viable and infective.

The concentration of larvae in the suspension was determined by placing an 0.1 ml aliquot onto a perspex slide marked with a grid, covering this with a cover slip and counting the number of larvae under a dissecting microscope.
FIGURE 2.1.
Faecal streaks on moist Whatman filter paper lining a petri-dish for culturing *N. dubius*
2.4. COLLECTION OF EXSHEATHED LARVAE OF N. DUBIUS

Exsheathed L₃ were obtained by the following procedure. Mice were starved overnight but allowed water. Third stage sheathed larvae of N. dubius were administered orally to these mice by introducing 10,000 L₃ in 1 ml of physiological saline into the lower part of the oesophagus using a blunt 19 gauge needle connected to a tuberculin syringe. After 30 minutes, the mice were killed by cervical dislocation. The stomach and intestine were removed, slit longitudinally and placed in a petri-dish containing physiological saline at 37°C. By this time the larvae had exsheathed in the stomach. After 30 minutes of incubation at 37°C, the suspension of exsheathed L₃ were filtered through a thin-layer of cloth in order to remove intestinal debris. The filtrate containing the exsheathed L₃ was kept on ice for a few minutes to immobilise them and then centrifuged at 500g for 1 minute. After centrifuging, the supernatant was discarded and the pellet collected. The exsheathed L₃ were finally separated from the pellet containing some intestinal debris by means of a sucrose gradient. The gradients were formed in 15 x 150 mm glass centrifuge tubes. Four ml of a 40% w/v sucrose solution was overlaid with 5 ml of a 10% w/v sucrose solution. One ml of the pellet containing the exsheathed L₃ was added and the tubes centrifuged at room temperature for 5 minutes at 1500g. After centrifuging, the exsheathed L₃ were removed from the interphase between the 40% and 10% sucrose solutions, washed in saline and finally resuspended in medium A. The larvae were kept at 4°C in this medium until required.

2.5. COLLECTION OF POST-INFECTIVE LARVAE

Post-infected larvae were obtained by the method previously described by Ey, Prowse and Jenkin, (1981). Mice received orally
2,500 $L_3$, suspended in 0.5 ml of physiological saline. They were killed by cervical dislocation 48 hours, 72 hours and 96 hours post infection. The entire length of the small intestine from just above the pyloric sphincter to the ileo-caecal valve was removed and freed of most of the connective tissue, taking special care not to cut or tear the intestinal wall. After tying the ends with surgical thread, to prevent extrusion of the luminal contents, each intestine was immersed in 15 ml of pre-warmed (37°C) medium (see below) in a 50 x 13 mm plastic petri-dish (Disposable Products Pty Ltd., South Australia) and incubated at 37°C for a period of 2 hours. The medium was a 1:4 mixture of medium 199 (Flow Laboratories, U.S.A.) and physiological saline and contained 200 I.U. per ml of both penicillin and streptomycin sulfate. At the end of the incubation period, the intestines were removed from the dishes and discarded. The larvae, which emerged through the wall of the intestine into the medium, were collected and washed by centrifuging in the same medium at 500g for 1 minute. Finally, they were resuspended in medium A and kept at 4°C until required. The concentration of emergent larvae in the suspension was determined by counting under a dissecting microscope (x16) the number in an 0.1 ml aliquot placed onto a perspex grid slide, covered with a coverslip. The number of emergent larvae usually recovered was c.a. 10% of the orally administered dose.

Adult worms were collected from mice which had been infected orally with 200 $L_3$ 14 days previously. The mice were killed by cervical dislocation, the intestines removed, slit longitudinally and placed in a petri-dish containing physiological saline at 37°C. The adult worms became very active, moved away from the intestines and could be removed with a pasteur pipette and counted (Fig. 2.2.).
FIGURE 2.2

Post-infective larvae obtained from the intestines of mice infected with *N. dubius* (2,500 L₃ per mouse) and killed at the indicated times after infection.

(a) 48 hours, (b) 72 hours and (c) 96 hours, (d) adult worms (14 days post-infection).

Magnification, x18.
2.6. **METHOD FOR IMMUNISING MICE AGAINST INFECTION WITH N. DUBIUS**

Mice were immunised routinely by administration of 2-4 doses of L₃ either orally or intravenously at 14 day intervals. In some experiments, mice were injected intravenously with 8 doses of 2,000 killed exsheathed L₃ in 0.2 ml of physiological saline. Seven day intervals separated each injection. Killed larvae were prepared by freezing and thawing twice.

2.7. **ASSAY OF RESISTANCE TO INFECTION WITH N. DUBIUS**

The level of resistance was determined by comparing the number of encysted larvae in the small intestine of immunised mice with the number seen in control animals following an oral challenge with L₃. The same batch of L₃ was used within each experiment. Cysts were counted on the 6th day after challenge in the following manner. The small intestine was removed from just below the pyloric sphincter to the ileo-caecal valve and pressed between two pieces of transparent perspex. Both sides of the intestine were then scanned under a dissecting microscope and the cysts counted (Fig. 2.3).

2.8. **COLLECTION OF SERUM**

Mice were bled from the retro-orbital plexus under ether anaesthesia and the blood was allowed to clot at room temperature for 30-60 minutes. It was then placed on ice, and the serum collected and held at 0°C. When serum was used as a source of complement, it was (unless otherwise stated) always used in experiments within 4-6 hours of bleeding the mice. Serum not used as a complement source was stored at -20°C. Inactivation of complement was carried out by heating the serum at 56°C for 30 minutes, or by methylamine treatment.
FIGURE 2.3.
Small intestine showing cysts from mice infected with $L_3$ 6 days previously.

C = cyst.
2.9. **INACTIVATION OF COMPLEMENT BY METHYLAMINE**

Serum complement was sometimes destroyed with methylamine which attacks a unique, labile thioester bond in C3, C4 and $\alpha_2$-macroglobulin by nucleophilic substitution (Porter and Sim, 1981). Samples of fresh NMS and IMS (2 ml) were mixed with 0.2 ml 1M methylamine-HCl/0.13M Sodium phosphate, pH 8.0 and incubated at $37^\circ$ for 90 minutes (cf. Pangburn, Schreiber and Müller-Eberhard, 1981). The sera were then dialysed at room temperature against 1 litre batches of PBS supplemented with 0.15 mM CaCl$_2$ and 0.5 mM MgCl$_2$ (4 changes; each 45 minutes). They were then tested immediately, together with the untreated sera which had been held on ice, in vitro larvicidal assay.

2.10 **COLLECTION OF PERITONEAL EXUDATE CELLS**

Peritoneal exudate cells were collected in the following manner. Mice were killed by cervical dislocation and the skin over the abdomen deflected. Two ml of ice cold heparin-medium B were then injected into the peritoneal cavity. The abdomen was massaged gently and the fluid withdrawn with a 22 gauge needle mounted onto a 2 ml syringe. The cells were then transferred to a siliconised tube on ice and washed twice in medium B, by centrifuging at 500 g for 5 minutes. Finally, the cells were resuspended in the above medium.

2.11. **CELL COUNTING AND VIABILITY**

The total number of cells in the above suspension was assessed by diluting a known volume of the cell suspension into a white cell counting fluid consisting of 0.1% gentian violet in 10% v/v acetic acid/distilled water. A haemocytometer was filled with an aliquot of this and the cells counted.

In order to determine the viability of the cells, an aliquot of the cell suspension was mixed with an equal volume of 0.1%
trypan blue in physiological saline and incubated at room temperature for three minutes. The viability of the cells was assessed by trypan-blue exclusion and only those cultures showing 90-95% viability were used.

2.12. PREPARATION OF SILICONISED GLASS TUBES

Siliconised glass tubes were prepared by boiling glass tubes in 1% 7x cleaning solution (Flow Laboratories, U.S.A.) for 10 minutes and washed 3 times in distilled water. These tubes were then treated with conc. HCl for 30 minutes, washed 3 times in distilled water, twice in acetone and twice in chloroform. They were then allowed to dry in an incubator at 120°C for 1 hour. After drying at this temperature silicone glass treatment solution (2% dimethyl dichlorosilane in CCl₄, (AJAX Chemicals, Aust.) was added to the sides of the tubes and the tubes dried at room temperature. Finally, the tubes were washed 3 times in distilled water.

2.13. TREATMENT OF MICE WITH SALMONELLA ENTERITIDIS 11 RX FOR THE COLLECTION OF ACTIVATED PERITONEAL EXUDATE CELLS

In order to obtain 'activated' macrophages mice were treated with Salmonella enteritidis 11 RX. The properties of this strain of bacteria have been described previously (Hardy and Kotlarski, 1971). An overnight culture of S. enteritidis 11 RX was diluted 1:10 in nutrient broth and incubated for a further 3 hours at 37°C with agitation. At the end of the incubation period, the log phase culture contained approximately 10⁹ organisms/ml as determined by viable counting. The broth culture was diluted in saline to give a concentration of bacteria of c.a. 2 x 10⁶/ml and 0.2 ml of this suspension injected intraperitoneally into mice. The peritoneal exudate cells were
collected 8 days later by the method described previously.

2.14 **MEASUREMENT OF HEMOLYTIC COMPLEMENT ACTIVITY**

The following assay was used to determine the titre of hemolytic complement in serum samples. Sheep erythrocytes were sensitised with antibody by slowly adding one volume of a 1:1,000 dilution of rabbit hemolysin (Commonwealth Serum Laboratories, Melbourne, Aust.) to an equal volume of a 0.14% v/v suspension of erythrocytes in VBS containing 0.2 mg/ml BSA (VBS-BSA). This mixture was incubated at 37° for 30 minutes. Serial two-fold dilutions of 0.2 ml of test sera were made in VBS-BSA in small glass tubes, and to each of these was then added 0.4 ml of sensitised erythrocytes and 0.2 ml of VBS-BSA. Two control tubes, one containing sensitised erythrocytes in VBS-BSA alone and the other sensitised erythrocytes in distilled water were included. The tubes were incubated in a 37° water bath for 60 minutes, and then centrifuged at 500 g for 5 minutes to remove unlysed cells. Haemoglobin release from lysed cells was determined by measuring the absorbance of each supernatant at 415 nm. The number of CH₅₀ units in the test sera was calculated as the inverse dilution of the sera giving 50% hemolysis, fifty percent hemolysis being the average between the absorbance at 415 nm of the controls of VBS-BSA and distilled water.

2.15 **CONCENTRATION OF SERUM OR IMMUNOGLOBULIN FRACTIONS BY ULTRAFILTRATION**

Samples were concentrated under N₂ (30 atmospheres) using Amicon stirred cells and Diaflo YM-10 ultrafiltration membranes (Amicon Corporation, U.S.A.).

2.16 **AMMONIUM SULPHATE PRECIPITATION OF IMMUNOGLOBULINS**

Immunoglobulins were precipitated at 0° from serum or fractions obtained by column chromatography by slowly adding solid ammonium
sulphate to 45% saturation (16.5 g/63 ml sample). The suspension was stirred at 4\(^\circ\) for 1-2 hours. After centrifugation at 12,000 g for 20 minutes at 4\(^\circ\), the supernatant was discarded and the pellet redissolved in saline and dialysed against PBS/0.15 mM Ca\(\text{Cl}_2\)/0.5 mM Mg\(\text{Cl}_2\).

2.17 PREPARATION OF PURIFIED MOUSE IMMUNOGLOBULINS

2.17.1. SEPHADEX G-200 CHROMATOGRAPHY

Forty millilitre aliquots of serum were fractionated at 4\(^\circ\) on a 100 x 5.5 cm column of Sephadex G-200 at a flow rate of 36 ml/hour. The fraction size was 18 ml. The eluent was 25 mM tris-HCl/132 mM NaCl/0.1 mM EDTA/8 mM Na\(_3\) citrate, pH 8.0. The absorbance of each fraction was measured at 280 nm and/or 295 nm to identify the major protein peaks (macroglobulins, IgG and albumin). These were pooled separately, concentrated by ultrafiltration and analysed by ELISA for IgM and IgG (see section 2.20).

2.17.2 PROTEIN A-SEPHAROSE CHROMATOGRAPHY

Mouse Ig\(_G_1\), IgG\(_{2a}\) and IgG\(_{2b}\) immunoglobulins were purified by affinity chromatography on protein-A-Sepharose 4B by Dr. P.L. Ey (Department of Microbiology & Immunology, University of Adelaide) as described by Ey, Prowse and Jenkin, (1978). Briefly, 4-5 ml of serum was mixed with 2 ml of 0.1M sodium phosphate pH 8.2 and applied to the protein A-Sepharose column (17 ml bed volume) which had been equilibrated with 0.1M sodium phosphate pH 8.2 at 4\(^\circ\). The column was washed at pH 8.2 until no more protein was recovered in the effluent as determined by absorbance at 280 nm. This was designated Pool 1 containing IgM and IgA. Pure Ig\(_G_1\), IgG\(_{2a}\) and IgG\(_{2b}\) immunoglobulins which had bound to the column were then recovered by sequential elution with 0.1M citrate buffers of pH 6.0, 4.5 and 3.5 respectively. Denaturation of the antibodies was minimized by adding
sufficient 1M Tris-HCl pH 9.0 to the collection tubes to ensure neutralization of the collected fractions. Four pools of serum proteins were thus obtained and these were analysed for IgM, IgG1, IgG2a and IgG2b by ELISA. Pool 1 contained IgM, IgA and IgE together with the bulk of the serum proteins; Pool 2 contained pure IgG1; Pool 3, pure IgG2a; and Pool 4, pure IgG2b. The protein concentration of the purified IgG immunoglobulins were calculated from the absorbance of the solutions at 280 nm, assuming an extinction coefficient (1% w/v; 1 cm) of 14.

2.18. PREPARATION OF PURIFIED MOUSE IgG and IgM IMMUNOGLOBULINS

For some experiments (Table 7.9) pure mouse IgG and IgM immunoglobulins were prepared by Dr. P.L. By using a combination of ammonium sulphate precipitation, gel filtration on Sephacryl S-200 and S-300, followed by chromatography on protein-A-Sepharose 4B (for IgG) or DEAE-Sephacel (for IgM).

2.19. RADIAL IMMUNODIFFUSION ANALYSES (OUCHTERLONY TEST)

Radial immunodiffusion tests (Ouchterlony, 1962) were carried out in 1% agarose (Seakem, HGT, U.S.A.) in tris-buffered saline, pH 8.0 (containing 0.1mM EDTA and 8mM NaN3). Six millilitres of molten agarose were poured onto a 7.5 x 5.0 cm glass slide pre-coated with 0.5% agar in distilled water. The agarose was allowed to solidify at 4°C for 30 minutes. Wells of 3.0 mm diameter were punched a few millimetres apart using a template and 10μl samples of either antigen or antibody were placed into opposing wells and allowed to diffuse toward one another in a moist chamber at 37°C for 48 hours. The slide was then pressed, washed in saline, dried and finally stained with 0.005% w/v Coomassie blue R-250 in an aqueous solution of 43%
ethanol/10% glacial acetic acid. Destaining was accomplished in an aqueous solution of 43% ethanol/10% glacial acetic acid.

2.20 **ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISA)**

The concentration of various immunoglobulin isotypes in serum or serum fractions was estimated using a competitive inhibition ELISA. The method was based on the ability of soluble immunoglobulin in the test sample to inhibit the binding of alkaline phosphatase conjugated, to affinity purified rabbit antibodies, specific for the heavy chain of the appropriate mouse immunoglobulin, to wells coated with purified mouse IgM, IgG\(_1\), IgG\(_{2a}\), IgG\(_{2b}\) or IgA.

The assays were performed in 96 well round-bottomed acrylic microtitre trays (Linbro, Flow Laboratories, U.S.A.). The wells were coated with purified mouse immunoglobulin by the addition of 80μl of a solution of the immunoglobulin (5μg/ml) in 25mM tris-buffered saline, pH 7.5 + 0.1% NaN\(_3\). After 1-2 hours at room temperature, the wells were washed twice with PBS containing 0.05% Tween 20 and 0.2mg/ml of BSA (washing buffer; 190μl per well). Eighty microlitres of washing buffer were then added to each well and the test sera and reference immunoglobulin solution (of known concentration) were each titrated in duplicate in serial 2-fold dilutions across the rows of the tray. The reference solution was used in order to construct a standard curve from which the concentration of immunoglobulin in the test samples could be determined. Control rows consisting of uncoated wells (negative control) and coated wells containing only washing buffer (positive control) were routinely included. To every well was then added 100μl of the appropriate enzyme-antibody conjugate (2 ng of antibody) in 20mM tris-HCl/0.15M NaCl/0.1mM MgCl\(_2\)/0.25μM ZnCl\(_2\)/0.2 mg/ml BSA/0.1% NaN\(_3\) (pH 7.5). The trays were covered and incubated overnight at room temperature. The wells were then washed...
twice with washing buffer and finally 160μl of substrate solution (1 mg/ml of disodium p-nitrophenylphosphate; Sigma Chemical Company, U.S.A.) in 1M diethanolamine - HCl/1mM MgCl₂/0.05% NaN₃ (pH 9.8) was added to each well. The trays were incubated at 37° for 4 hours, after which the amount of p-nitrophenol produced in each well was assessed spectrophotometrically by reading the absorbance at 405 nm, (micro Elisa Reader, MSE, Scientific Instruments, U.K.).

The results of a typical IgG₁ ELISA are shown in Fig. 2.4. The concentration of IgG₁ in the test sample was estimated by first calculating the amount of IgG₁ in the reference solution required to produce exactly 50% inhibition of colour (0.29 μg/ml). This amount of IgG₁ was then taken as that present in the test sample at the dilution giving 50% inhibition (1/51,984). The concentration of IgG₁ in the undiluted sample was then calculated (15.08 mg/ml).

2.21. ADSORPTION OF IMMUNOGLOBULIN FROM IMMUNE MOUSE SERUM BY AFFINITY CHROMATOGRAPHY

Immunoglobulins were removed from immune mouse serum by passing the serum through an anti-mouse F(ab')₂-Sepharose column (provided by Dr. P.L. Ey; bed volume 5 ml) which had a capacity for 60 mg of mouse immunoglobulins. Serum was obtained from mice bled 10 days after the final of 4 doses of 200 N. dubius L₃ given orally 14 days apart. A 6 ml sample of undiluted serum was applied to the anti-immunoglobulin column at 4°. The column was washed with PBS and 1 ml fractions were collected. These were analysed for protein by measuring their absorbance at 280 nm. The fractions containing the bulk of the effluent serum proteins were pooled to give an immunoglobulin-depleted serum fraction of 14 ml,
FIGURE 2.4.

IgG₁ ELISA. Serial 2-fold dilutions of samples were made, for purified IgG₁ starting from 2.5 µg/ml and for the test serum sample, 1/1,000. (a) and (b) represent the negative and positive controls respectively. The amount of IgG₁ required to produce 50% inhibition of colour was 0.29 µg/ml ($= 2.5 \times \frac{1}{2.5}$) for the reference solution. The concentration of IgG₁ in the test sample was calculated to be 15.08 µg/ml ($= 0.29 \times 1000 \times 2^{5.7}$).
2.22. **ADSORPTION OF COMPLEMENT COMPONENT C3 FROM MOUSE SERUM BY AFFINITY CHROMATOGRAPHY**

Five millilitres of fresh immune mouse serum was depleted of the third component of complement (C3) in a manner similar to that described above for immunoglobulin depletion, using an anti-mouse C3-Sepharose immuno-adsorbent column. The anti-C3 column was kindly provided by Dr. P.L. Ey, who prepared it using mouse C3 purified by the method of Tack and Prahl, (1976). Three millilitre fractions were collected and analysed by absorbance at 280 nm. Selected fractions were pooled to yield a C3-depleted serum (4.7 ml). This was analysed for C3 by ELISA and shown to contain less than 0.025% of the C3 present in the original unadsorbed serum.

2.23. **STATISTICS**

Data were analysed by the Kruskal-Wallis one-way analysis of variance and the Mann-Whitney U test (Siegel, 1956). A probability of 0.05 or less was considered as being significantly different.
CHAPTER 3

Factors involved in the adherence of peritoneal exudate cells to the larval and adult stages of *N. dubius*. 
3.1. INTRODUCTION

Earlier work had established unambiguously that the infective L₃ are destroyed after penetrating the muscularis mucosa of the resistant host and not as had been previously suggested by a mechanism involving expulsion from the small intestine prior to penetration (Chaicumpa et al., 1977a). Further in vivo studies using millipore chambers implanted into the peritoneal cavity of mice resistant to re-infection showed that the peritoneal exudate cells of these mice have a marked effect on the infectivity of the L₃. Damage to the larvae occurred only if the cells came into direct contact with the cuticle. The cells associated with the cuticle of the larvae recovered from the peritoneal cavities of immune mice appeared to be predominantly macrophages (Chaicumpa, Jenkin and Fischer, 1977b). Since adhesion of the cells to the surface of the parasite appeared to be an important pre-requisite before killing took place it seemed important to study the factors involved in promoting adhesion in the hope it might lead to a better understanding of the mechanisms of acquired immunity to not only this parasite, but possibly other parasitic nematodes.

3.2 METHOD

ASSAY FOR THE ADHERENCE OF PERITONEAL EXUDATE CELLS TO DIFFERENT DEVELOPMENTAL FORMS OF N. DUBIUS

This assay was carried out in sterile 15 x 100 mm siliconised glass tubes with a screw cap. To each tube 100μl of serum diluted to a concentration of 1:1.5 were added. The diluent was either medium A or VBS containing where indicated in the text 10mM EDTA or EGTA. To these tubes 500 larvae or 50 adult worms in 100μl of the appropriate diluent (i.e. that used to dilute the serum) were added.
At this stage the final dilution of serum was 1:3. The contents of
the tubes were mixed well and incubated at 37°C for 90 minutes with
gentle agitation. This mixture was then diluted with 5 ml of
physiological saline and spun at 500g for 1 minute. The supernatant
was withdrawn leaving the pelleted larvae in approximately 200μl.
This washing step was repeated using medium A. Finally 250μl of a
washed peritoneal exudate cell suspension from normal or immune mice,
(collected as described previously) containing 5 x 10⁵ cells in
medium B, were added. The contents of the tubes were mixed and
incubated at 37°C for 1 hour, after which the larvae or adult worms
were counted for adherence of cells in the following manner.

Slides coated with bovine serum albumin (BSA) were prepared
by dipping them into a 7% solution of BSA in distilled water and
allowing them to dry at room temperature. A drop of the test
suspension of larvae and cells was placed onto a slide and a drop of
Leishman's stain added and mixed well. A cover-slip was then mounted
onto the slide and the larvae or adult worms were examined under a
microscope (x400), enabling the number of cells adhering to each larva
or adult worm to be counted. Thirty parasites from each tube were
counted.

Control tubes, which contained cells incubated with
unsensitised larvae or adult worms were also included in this assay.

3.3. RESULTS
3.3.1. FACTORS INVOLVED IN THE ADHERENCE OF PERITONEAL
        EXUDATE CELLS FROM NORMAL MICE TO EXSHEATHED L₃

Although it had been shown previously that peritoneal exudate
cells from normal mice are unable to damage L₃ even in the presence
of immune serum (Chaicumpa et al., 1977b) it seemed important to
establish whether the initial step in this cell-parasite interaction (i.e. adhesion) was initiated by similar factors irrespective of the immune status of the host from which they were derived.

Using the above assay exsheathed L3 were sensitised under various conditions as indicated in Table 3.1. with freshly collected serum from either normal mice or mice immune to re-infection. It is clear from the data that in the absence of serum phagocytic cells do not adhere to the cuticle of the exsheathed L3. However exposure to the parasites to NMS in VBS i.e. in the presence of Ca++ and Mg++ ions promoted the adherence of large numbers of cells. The fact that adhesion was abolished if the larvae were sensitised with NMS in the presence of EDTA but not if the larvae were sensitised with serum in the presence of EGTA indicated that the cells were binding to the larvae via C3 and confirms previous observations that parasites may activate complement via the alternative pathway (Prowse, Ey and Jenkin, 1979b). In contrast cells adhered strongly to exsheathed L3 sensitised with IMS(4) in the presence of EDTA although the mean number of cells adhering to each larva was significantly lower than that observed adhering to larvae sensitised with IMS(4) in VBS. It was found that more than 95% of cells adhering to the larvae were macrophages (Table 3.2 and Fig. 3.1). These results suggested that macrophages may bind to the cuticle of the exsheathed L3 via antibody the Fc receptors on their membrane as well as via C3 receptors. These experiments were repeated on two separate occasions with similar results.

The above experiment was repeated using both peritoneal exudate cells from normal mice and from mice resistant to this infection in order to see if the factors involved in adherence of these cells to the surface of the parasite were similar. Peritoneal exudate cells were collected from normal and immune mice as described previously, washed
The ability of serum from normal mice and mice immune to re-infection with *N. dubius* to promote the adherence of normal peritoneal exudate cells to exsheathed L₃ under various conditions.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Unsensitised larvae in</th>
<th>Larvae sensitised with 1:3 NMS in</th>
<th>Larvae sensitised with 1:3 IMS(4)* in</th>
</tr>
</thead>
<tbody>
<tr>
<td>199</td>
<td>0</td>
<td>52.5 ± 2.8</td>
<td>54.5 ± 2.4</td>
</tr>
<tr>
<td>VBS</td>
<td>0.03 ± 0.04</td>
<td>40.0 ± 2.6</td>
<td>68.3 ± 1.4</td>
</tr>
<tr>
<td>VBS-EDTA**</td>
<td>0.03 ± 0.04</td>
<td>0</td>
<td>32.4 ± 3.1</td>
</tr>
<tr>
<td>VBS-EGTA**</td>
<td>0</td>
<td>43.4 ± 2.5</td>
<td>80.6 ± 2.2</td>
</tr>
</tbody>
</table>

* Serum from mice bled on day 14 following 4 doses of 200L₃ orally on days -42, -28, -14 and 0

** EDTA and EGTA at a final concentration of 10mM
TABLE 3.2.

Total number of normal peritoneal exudate cells and percentage of various cell types adhering to each larva which had been sensitised with serum from normal mice or mice immune to re-infection with N. dubius under various conditions.

<table>
<thead>
<tr>
<th>Treatment of exsheathed L₃</th>
<th>Sensitising medium</th>
<th>Total cell adhered</th>
<th>% of total cell adhered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>macrophages</td>
<td>lymphocytes</td>
</tr>
<tr>
<td>None</td>
<td>VBS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>VBS-EDTA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>VBS-EGTA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1:3 NMS</td>
<td>VBS</td>
<td>54.5±1.4</td>
<td>96.4±0.5</td>
</tr>
<tr>
<td></td>
<td>VBS-EDTA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>VBS-EGTA</td>
<td>53.4±2.9</td>
<td>97.3±0.4</td>
</tr>
<tr>
<td>1:3 IMS(4)*</td>
<td>VBS</td>
<td>55.7±1.9</td>
<td>96.8±0.4</td>
</tr>
<tr>
<td></td>
<td>VBS-EDTA</td>
<td>23.6±2.7</td>
<td>95.3±0.5</td>
</tr>
<tr>
<td></td>
<td>VBS-EGTA</td>
<td>54.9±2.1</td>
<td>96.3±0.6</td>
</tr>
</tbody>
</table>

* Serum from mice bled on day 14 following 4 doses of 200 L₃ orally on day -42, -28, -14 and 0.
FIGURE 3.1.
Adherence of normal peritoneal exudate cells to exsheathed $L_3$ which had been sensitised with (a) IMS(4) in the presence of VBS; (b) IMS(4) in the presence of VBS-EDTA; and (c) unsensitised larvae in VBS.
by centrifuging at 500g for 5 minutes and finally resuspended to a concentration of \(2 \times 10^6\) /ml. Exsheathed \(L_3\) were sensitised with either serum from normal mice or immune mice under similar conditions outlined in the previous experiment. After washing the \(L_3\), peritoneal exudate cells were added and the number of adherent cells scored as before. The data given in Table 3.3 show that the factors promoting adherence of the macrophages to the parasite are similar irrespective of whether they were obtained from normal or immune mice. These results are at variance with previous results where it had been shown that macrophages \textit{in vitro} from mice resistant to infection were able to damage the parasite in the absence of specific antibody and/or complement (Chaicumpa et al., 1978; Prowse et al., 1979b). Previous investigations had shown also that damage to the parasite by macrophages required intimate contact between the cell and the cuticle of the parasite (Chaicumpa et al., 1977b). A possible reason for the discrepancy between the present results and those previously described will be discussed later. However in view of the fact that the data indicate that there was no difference between normal peritoneal exudate cells and those from animals resistant to infection, in their requirements for cell adhesion cells from normal animals were used in the following experiments for the sake of expediency.

3.3.2. \textbf{THE ABILITY OF SERUM FROM NORMAL MICE OR MICE RESISTANT TO RE-INFECTION TO PROMOTE THE ADHERENCE OF NORMAL PERITONEAL EXUDATE CELLS TO DIFFERENT DEVELOPMENTAL FORMS OF N. DUBIUS.}

Following penetration of the muscularis mucosa of the small intestine the exsheathed \(L_3\) grow rapidly and moult once more around 90 hours post-penetration prior to their passage back into the lumen as adult worms.
TABLE 3.3
The adherence of peritoneal exudate cells from immune or normal mice to exsheathed $L_3$ promoted by serum from normal or resistant mice.

<table>
<thead>
<tr>
<th>Source of peritoneal exudate cells</th>
<th>Treatment of larvae</th>
<th>Mean number of adherent cells on each exsheathed $L_3$ $(\bar{x} \pm SE, n=30)$ sensitised in VBS</th>
<th>VBS-EDTA</th>
<th>VBS-EGTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal mice</td>
<td>none</td>
<td>0.2±0.1</td>
<td>0.4±0.2</td>
<td>0.1±0.04</td>
</tr>
<tr>
<td></td>
<td>1:3 NMS</td>
<td>57.2±2.5</td>
<td>0.5±0.2</td>
<td>43.0±2.4</td>
</tr>
<tr>
<td></td>
<td>1:3 IMS(4)</td>
<td>56.1±2.3</td>
<td>18.2±2.2</td>
<td>48.0±2.0</td>
</tr>
<tr>
<td>immune* mice</td>
<td>none</td>
<td>0.4±0.2</td>
<td>0.4±0.2</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td></td>
<td>1:3 NMS</td>
<td>45.5±2.8</td>
<td>0.4±0.1</td>
<td>54.1±1.9</td>
</tr>
<tr>
<td></td>
<td>1:3 IMS(4)</td>
<td>46.5±2.6</td>
<td>14.7±1.5</td>
<td>42.3±2.9</td>
</tr>
</tbody>
</table>

* Serum from mice bled on day 16 following 4 doses of 200 $L_3$ orally on day -42, -28, -14 and 0. The peritoneal exudate cells from these mice were also collected on the day of bleeding.

** EDTA and EGTA at a final concentration of 10mM.
In view of these changes it was of interest to see if cell adhesion to the larvae following penetration and also to the adult worms was promoted by similar factors as those involved in the adhesion of cells to exsheathed $L_3$.

Exsheathed $L_3$ (2,500) were fed orally to mice and at different intervals of time post-infective parasites were collected according to the method outlined previously (see Section 2.5). Following the experimental protocol outlined above the various developmental stages were sensitised with NMS or IMS(4) in the presence of VBS, VBS-EDTA and VBS-DGTA. Following sensitisation the larvae were washed and $5 \times 10^5$ peritoneal exudate cells were added. After the appropriate period of incubation the number of cells adhering to the various developmental stages were counted as before. Control tubes contained parasites treated in the same manner with relationship to the buffers but in the absence of serum. The experiment was designed such that all stages were tested on the same day. It is clear from the data given in Table 3.4 that all of the various stages of the parasite were capable of activating complement by the alternative pathway with the marked exception of larvae collected 96 hours post-infection. This activation of complement promoted the binding of peritoneal exudate cells to the cuticle of the parasite presumably through C3 and it's receptor on the membrane of the phagocytic cell. It is also of some interest to note that the number of cells binding to the larvae promoted by this particular complement component appeared to decrease quite markedly up to 96 hours post-infection when it was almost nil. It is also clear that peritoneal exudate cells were able to bind to all larval stages and to adult worms via their Fc receptors. Again it is interesting to note that binding of cells to 96 hours post-infective larvae sensitised with IMS(4) appears
TABLE 3.4.
The ability of sera from normal mice and mice immune to re-infection to promote the adherence of normal peritoneal exudate cells to different developmental forms of *N. dubius*.

<table>
<thead>
<tr>
<th>Developmental form of <em>N. dubius</em></th>
<th>Sensitising serum</th>
<th>Mean number of adherent cells on each larva ($\bar{X} \pm SE, n=30$) sensitised in the presence of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>VBS</strong></td>
</tr>
<tr>
<td>Sheathed <em>L</em>$_3$</td>
<td>none</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td></td>
<td>1:3 NMS</td>
<td>77.0±1.4</td>
</tr>
<tr>
<td></td>
<td>1:3 IMS(4)*</td>
<td>78.6±2.3</td>
</tr>
<tr>
<td>Exsheathed <em>L</em>$_3$</td>
<td>none</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1:3 NMS</td>
<td>78.0±2.1</td>
</tr>
<tr>
<td></td>
<td>1:3 IMS(4)</td>
<td>86.5±1.6</td>
</tr>
<tr>
<td>48 hours post-infective larvae</td>
<td>none</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td></td>
<td>1:3 NMS</td>
<td>58.9±4.2</td>
</tr>
<tr>
<td></td>
<td>1:3 IMS(4)</td>
<td>70.0±4.2</td>
</tr>
<tr>
<td>72 hours post-infective larvae</td>
<td>none</td>
<td>0.7±0.3</td>
</tr>
<tr>
<td></td>
<td>1:3 NMS</td>
<td>75.0±9.0</td>
</tr>
<tr>
<td></td>
<td>1:3 IMS(4)</td>
<td>112.5±11.0</td>
</tr>
<tr>
<td>96 hours post-infective larvae</td>
<td>none</td>
<td>0.9±0.4</td>
</tr>
<tr>
<td></td>
<td>1:3 NMS</td>
<td>5.3±0.8</td>
</tr>
<tr>
<td></td>
<td>1:3 IMS(4)</td>
<td>36.8±6.3</td>
</tr>
<tr>
<td>Adult worms</td>
<td>none</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td></td>
<td>1:3 NMS</td>
<td>48.6±8.1</td>
</tr>
<tr>
<td></td>
<td>1:3 IMS(4)</td>
<td>48.0±7.0</td>
</tr>
</tbody>
</table>

* Serum from mice bled on day 14 following 4 doses of 200LD$_9$ orally on day -42, -28, -14 and 0.
to be due entirely to binding via their Fc receptors. This experiment was repeated with similar results, the 96 hours post-infective larvae again not activating complement via the alternate pathway.
CHAPTER 4

The ability of different immunoglobulin isotypes to promote the adhesion of normal peritoneal exudate cells to *N. dubius.*
4.1. INTRODUCTION

Mice infected two or more times with 100-200 L3 develop a high degree of immunity to subsequent re-infection (van Zandt, 1961; Panter, 1969; Bartlett et al., 1972; Chaicumpa et al., 1977a). One of the features of repeated infection is a remarkable increase in the level of IgG1 (Crandall et al., 1974; Prowse et al., 1978b). Levels have been reported as high as 35 mg/ml, an extraordinary level comparable with concentrations in mice carrying IgG1 plasmacytomas. Unusually high IgG1 levels have been observed in a number of chronic infections caused by metazoan parasites (Mitchell et al., 1977a; Mitchell et al., 1977b; Sher et al., 1977). The immunological importance of this IgG1 increase is unclear but it has been suggested that it may be parasite protective rather than host protective since IgG1 is putatively unable to fix complement (Mitchell et al., 1977a; Nussenzweig, Merryman and Benacerraf, 1984). However more recently it has been reported that IgG1 may indeed fix complement (Ey, Prowse and Jenkin, 1979). Since cell adhesion is an important pre-requisite which may lead to loss of infectivity of the larvae (Chaicumpa et al., 1978) it was important to compare the efficacy of the various immunoglobulin isotypes in promoting this reaction.

4.2. RESULTS

4.2.1. THE EFFICACY OF DIFFERENT IMMUNOGLOBULIN ISOTYPES IN PROMOTING THE ADHERENCE OF NORMAL PERITONEAL EXUDATE CELLS TO EXSHEATHED L3

Serum obtained from mice immunised four times as reported in Materials and Methods was fractionated on a Protein A sepharose column to obtain the various IgG isotypes namely IgG1, IgG2a and IgG2b. The effluent was also used in these studies and consisted mainly of the
isotype IgM (0.812 mg/ml) contaminated with IgA (0.12 mg/ml) and some IgG1 (0.015 mg/ml) as determined by ELISA. Following the protocol given in Chapter 3, 2-fold dilutions of each isotype were made in VBS + 10mM EDTA such that the starting concentration of IgG1 was 22 mg/ml; IgG2a, 11.5 mg/ml; IgG2b, 9.2 mg/ml and the effluent consisting of mainly IgM, 0.4 mg/ml. The physiological level of the various isotypes in the serum pooled from the above mice was as follows, IgG1 (15.075 mg/ml), IgG2a (1.5 mg/ml), IgG2b (0.4 mg/ml) and IgM (0.12 mg/ml). To the various concentrations of the isotypes exsheathed L3 were added and the experimental protocol followed that given in Chapter 3 for assaying normal and immune serum for their adherence promoting properties. The data presented in Table 4.1 show that all the isotypes contained antibody capable of promoting adherence of macrophages to the surface of the exsheathed L3. However it should be noted that at a physiological level only two isotypes IgG1 and IgM enhanced adherence.

4.2.2. THE ABILITY OF DIFFERENT IMMUNOGLOBULIN ISOTYPES TO PROMOTE THE ADHERENCE OF NORMAL PERITONEAL EXUDATE CELLS TO DIFFERENT DEVELOPMENTAL FORMS OF N. DUBIUS

The above experiment was repeated using pre- and post-infective larvae and adult worms. However in this particular experiment a standard amount of a particular isotype in VBS + 10mM EDTA was used to sensitize the various stages. The concentrations of the isotypes were chosen from the preceding experiment as those inducing optimal cell binding and were as follows; IgG1 (22 mg/ml), IgG2a (5.8 mg/ml), IgG2b (4.6 mg/ml), IgM (0.4 mg/ml). Control tubes containing each of the parasite stages were set up in VBS-EDTA in the absence of
TABLE 4.1.
The efficacy of different immunoglobulin isotypes in promoting the adherence of normal peritoneal exudate cells to exsheathed L₃.

<table>
<thead>
<tr>
<th>Adherence promoted by</th>
<th>Amount of Ig isotype mg/ml</th>
<th>Mean number of adherent cells on each exsheathed L₃ (X ± SE, n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG₁</td>
<td>22.0</td>
<td>36.9±4.6</td>
</tr>
<tr>
<td></td>
<td>11.0</td>
<td>17.4±2.4</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>6.8±1.2</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>4.3±0.8</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>IgG₂a</td>
<td>11.5</td>
<td>34.0±3.4</td>
</tr>
<tr>
<td></td>
<td>5.8</td>
<td>28.1±2.3</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>8.9±1.2</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>5.1±0.8</td>
</tr>
<tr>
<td>IgG₂b</td>
<td>9.2</td>
<td>40.2±3.5</td>
</tr>
<tr>
<td></td>
<td>4.6</td>
<td>40.5±3.2</td>
</tr>
<tr>
<td></td>
<td>2.3</td>
<td>26.6±3.5</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>4.8±0.8</td>
</tr>
<tr>
<td>IgM</td>
<td>0.4</td>
<td>14.1±1.6</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>14.4±1.4</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>7.0±1.0</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>7.5±1.0</td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>4.1±0.6</td>
</tr>
</tbody>
</table>

Sensitisation was performed in VBS containing 10mM EDTA
immunoglobulin. Following the previously outlined experimental procedure the cells adhering to thirty parasites representing each of the various stages were scored. The experiment was arranged such that cell adhesion to all stages was measured on the same day. The data expressed in Fig. 4.1 show that the ability of IgM to promote the binding of peritoneal exudate cells to the larval stages increased dramatically from the pre-infective stages (i.e. the sheathed and exsheathed L₃) up to the 96 hour post-infective stage, but promoted little binding to the adult worms. The IgG₁ displayed a similar pattern of activity although binding of macrophages was considerably reduced when one used 96 hour post-infective larvae. It is interesting to note that the IgG₂a and IgG₂b appeared to play as an important role as the other two isotypes in enhancing binding of cells to the pre-infective larvae and possibly more so to the adult worms. However their role in this reaction was progressively diminished compared with IgG₁ and IgM, when one used post-infective larvae. The implication of these results will be discussed later. This experiment was repeated and gave similar results.

4.2.3. COMPARISON BETWEEN THE ABILITY OF KILLED L₃ AND LIVING L₃ TO PROVOKE THE PRODUCTION OF ANTIBODIES ENHANCING ADHESION OF NORMAL PERITONEAL EXUDATE CELLS TO EXSHEATHED L₃

There are numerous reports in the literature showing that mice may be effectively immunised against N. dubius infection by using living L₃ but not by using killed L₃ (see literary Review pg. 25). One possible reason for the inability of killed larvae to induce a
FIGURE 4.1.

Showing mean ± standard error of the number of cells bound to each stage of the parasite sensitised with purified IgM (0.4 mg/ml), IgG1 (22 mg/ml), IgG2a (5.8 mg/ml) and IgG2b (4.6 mg/ml).
state of immunity was that they failed to evoke antibodies which were capable of promoting adhesion of effector cells to the cuticle of the larvae. In order to test this possibility the following experiment was carried out.

Mice were divided into 3 groups of 10. Mice in group 1 were infected orally with 4 doses of 200 living sheathed $L_3$ 14 days apart and bled 5 days after the first dose and at 10 day intervals thereafter. Mice in the second group were injected intravenously with seven weekly doses of killed exsheathed $L_3$ and bled at the same time intervals as above. The killed larvae were prepared by freezing and thawing twice. Animals in the third group serving as controls received no treatment but were bled at the same time. All serum samples were tested for their ability to mediate cell adherence to exsheathed $L_3$. The larvae were sensitised with dilutions of serum (1/3, 1/30 and 1/300) in the presence of 10mM EDTA. The experimental protocol was similar to that given in Section 3.2. The results of this experiment presented in Table 4.2 show that killed larvae are capable of evoking antibodies which mediate cell adhesion. However what is interesting is that despite giving large numbers of killed larvae the titre of antibodies enhancing adhesion did not reach over the time period studied the levels observed in the serum from mice given living $L_3$ orally. In the latter group antibody demonstrated by the above method could be detected in the serum 25 days following the first oral feeding i.e. at a time when they had received a total of 400 larvae. In contrast in the group receiving killed larvae, antibodies were not detected until 35 days after the first dose at which time the mice had received a total of 10,000 larvae.
TABLE 4.2.
The ability of serum from mice immunised with killed exsheathed $L_3$ compared to mice immunised with living $L_3$ in promoting cell adherence.

<table>
<thead>
<tr>
<th>Type of immunisation</th>
<th>Day of bleeding</th>
<th>No. of adherent cells on each exsheathed $L_3^*$ sensitised with serum at a dilution of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:3</td>
</tr>
<tr>
<td>Control (non-immunised mice)</td>
<td>5</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>0.9±0.2</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>Living $L_3$ orally+</td>
<td>5</td>
<td>0.9±0.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5.5±1.2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>39.9±2.4</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>41.0±1.7</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>46.2±2.9</td>
</tr>
<tr>
<td>Killed exsheathed $L_3$, IV#</td>
<td>5</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>1.6±0.5</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>33.2±2.0</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>36.6±3.4</td>
</tr>
</tbody>
</table>

* Assayed as described (in Section 3.2) in VBS+10mM EDTA and expressed as mean±standard error of the number of adherent cells from 30 larvae.
+ Mice were immunised orally with 4 doses of $200L_3$ 14 days apart and bled 5 days after the first dose and at 10 day intervals thereafter.
# Mice were immunised intravenously with 7 weekly doses of 2,000 killed exsheathed $L_3$ and bled 5 days after the first dose and at 10 day intervals thereafter.
4.2.4. THE INABILITY OF KILLED LARVAE TO PROTECT MICE AGAINST INFECTION WITH N. DUBIUS.

The previous experiment demonstrated that killed larvae given in large numbers could stimulate the production of antibodies which mediate cell adherence. Despite previous reports showing that killed larvae were ineffective in inducing immunity it was felt worthwhile to attempt once more to induce resistance using killed vaccines since the doses given in previous experiments were much lower than those given in the above experiment. Consequently a group of eight mice were given eight intravenous injections of 2,000 killed larvae at weekly intervals and challenged orally 7 days after the last immunising injection with 200 L3. A control group of animals which had received no treatment was challenged in a similar fashion. On day 6 the mice were killed and the number of cysts in the muscularis mucosa of the small intestine counted. There was no significant difference in the number of cysts between the two groups being 166.2 ± 6.3 in the immunised group and 145.0 ± 9.1 in the control.
CHAPTER 5

The effect \textit{in vitro} of peritoneal exudate cells from immune mice on the infectivity of $L_3$ of \textit{N. dubius} sensitised with either specific antibody and/or complement.
5.1. INTRODUCTION

Previous studies by Chaicumpa et al., (1978) have shown that in vitro, peritoneal exudate cells of mice immune to infection with *N. dubius* have a marked effect on the infectivity of the L₃. In contrast, peritoneal exudate cells from unimmunised mice were unable to damage the larvae even in the presence of serum from immune animals.

It is also clear from the results given in Chapter 3, that peritoneal exudate cells from mice resistant to re-infection with this parasite were able to adhere to the cuticle of exsheathed L₃ either via their Fc or C3 receptors. The following experiments were carried out to define more clearly the involvement of antibody and/or complement in mediating damage to the L₃ by macrophages from resistant animals.

5.2. METHOD

**IN VITRO ASSAY FOR THE EFFECT OF PERITONEAL EXUDATE CELLS ON THE INFECTIVITY OF EXSHEATHED L₃**

The method described below was used in all in vitro experiments.

The assay was carried out in sterile 15 x 100 mm round-bottomed siliconised glass tubes with a screw cap. Exsheathed L₃ washed twice in medium A and suspended in the same at a concentration of 1,000 L₃/ml were used. To each tube were added 0.2 ml of the larval suspension. In addition were added 50 µl of either fresh or heat-inactivated serum from immune or normal mice. The tubes were then incubated at 37°C for 90 minutes, after which time peritoneal exudate cells, prepared as described previously, were added in 0.5 ml of medium B. The number of cells added was approximately 2 x 10⁶ per tube.
The tubes were then incubated at 37° for 1 hour. After this period of incubation, a further 5 ml of medium B were added, and each tube was gassed with 5% CO₂/air mixture for a few seconds and capped tightly. The tubes were then incubated at 37° for 48 hours. At the end of this time the tubes were centrifuged at 500 g for 1 minute and the supernatant withdrawn leaving the pelleted larvae in approximately 0.6 ml. The contents of each tube were carefully removed and fed to mice, the contents of one tube being fed to one mouse. The control groups contained larvae in medium B only. The results were assessed by counting the number of cysts in the intestine of the mice 6 days later and are expressed as the mean number of cysts in the 6 mice.

5.3. RESULTS

5.3.1. THE EFFECT OF INCUBATING PERITONEAL EXUDATE CELLS FROM RESISTANT MICE FOR DIFFERENT PERIODS OF TIME WITH EXSHEATHED L₃ ON THEIR INFECTIVITY AFTER SENSITISATION WITH IMS(2)

Initially experiments were carried out to determine the optimal time of incubation required for the peritoneal exudate cells from immune mice to produce an effect upon larval infectivity.

Tubes were divided into four groups of twelve and 0.2 ml of medium A containing 200 exsheathed L₃ were added to each tube. Group 1 serving as a control contained medium A and larvae only. To each tube in group 2 and 4 were added 50 μl of fresh serum from mice immunised twice orally with 200 L₃, IMS(2); whilst to group 3, 50 μl of medium A. All tubes were then incubated at 37° for 90 minutes after which time 2 x 10⁶ peritoneal exudate cells from mice given 2 immunising doses were added to each tube in group 3 and 4,
where as to groups 1 and 2, medium B only was added. After incubation at 37\(^\circ\) for 1 hour to allow time for the cells to adhere, all tubes were supplemented with a further 5 ml of medium B and the mixtures, after gassing with a 5% CO\(_2\)/air mixture, incubated at 37\(^\circ\) for 24 and 48 hours respectively. At the end of each incubation time, 6 tubes from each group were taken and centrifuged in order to leave the pelleted larvae in approximately 0.6 ml. Four groups of six mice representing the four groups of tubes were set up and following resuspension of the larvae, each mouse in each particular group received the contents of one tube. In addition a further control group of six mice were each given 200 exsheathed L\(_3\) suspended in 0.6 ml of medium B that had not been subjected to incubation. The results of this experiment are given in Table 5.1. There was no loss of infectivity of unsensitised larvae in the absence of peritoneal exudate cells over the incubation time studied. Neither was the infectivity of unsensitised larvae lost when incubated in the presence of cells alone nor of larvae sensitised with IMS(2) and incubated in the absence of cells. In contrast the infectivity of the sensitised larvae was significantly reduced following incubation with peritoneal exudate cells over a period of 48 hours but not after 24 hours. The experiment was repeated with similar results and in all future experiments of this nature 48 hours was chosen as the standard incubation time.

5.3.2. **THE EFFECT IN VITRO OF PERITONEAL EXUDATE CELLS FROM MICE GIVEN 2 IMMUNISING DOSES ON THE INFECTIVITY OF EXSHEATHED L\(_3\) SENSITISED WITH NMS OR IMS(2) TREATED OR UNTREATED AT 56\(^\circ\)**

The previous experiment showed that following 48 hours of
### TABLE 5.1

The effect of incubating peritoneal exudate cells from resistant mice for different periods of time with exsheathed $L_3$ on their infectivity after sensitisation with IMS(2).

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Sensitising Serum</th>
<th>$L_3$ with</th>
<th>Time of incubation (hours)</th>
<th>Number of cysts# (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>medium</td>
<td>0</td>
<td>162.5±9.7, 154.5±9.9, 151.7±5.1</td>
</tr>
<tr>
<td>2</td>
<td>IMS(2)(\Delta)</td>
<td>medium</td>
<td>24</td>
<td>N.D., 149.0±6.7, 143.3±6.2</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>cells</td>
<td>48</td>
<td>N.D., 158.3±8.0, 147.3±4.6</td>
</tr>
<tr>
<td>4</td>
<td>IMS(2) unheated</td>
<td>cells</td>
<td>0</td>
<td>123.8±8.0, 103.2±7.8</td>
</tr>
</tbody>
</table>

\(\Delta\) Serum was obtained from mice bled on day 10 after the final of 2 doses of 200 $L_3$ of *N. dubius* given orally 14 days apart.

# : At 24 hours incubation, analysed by Kruskall-Wallis one-way analysis of variance
1 vs 2 vs 3 vs 4, not significant

: At 48 hours incubation, analysed by Kruskall-Wallis one-way analysis of variance
1 vs 2 vs 3 vs 4, p<.01;
then analysed further by Mann-Whitney U test
1 vs 2 or 3, not significant
1 vs 4, p=.004

** Significant at $\alpha=.01$
incubation in vitro peritoneal exudate cells from mice given 2 oral
immunising doses reduced the infectivity of L_3 sensitised with serum,
from these immunised mice. The data obtained in Chapter 3 (adherence
assay) indicated also that both complement alone and antibody alone
promoted the adherence of peritoneal exudate cells from immune mice
to L_3. Since as mentioned earlier adhesion of the cells to the
cuticle of the larvae was necessary before damage occurred, it seemed
important to establish whether in the presence of complement alone
cells were able to damage the larvae as measured by a loss of infectivity.

The details of this experiment are outlined below. Peritoneal
exudate cells from mice given 2 immunising doses of L_3 orally or from
normal mice were collected as described earlier and resuspended in
medium B at a concentration of 4 x 10^6 cells/ml. Sera obtained
from normal mice or immunised mice were used in the latter case either
untreated or heat-inactivated at 56°. Tubes containing 200 exsheathed
L_3 in 0.2 ml of medium A were prepared and divided into nine groups
of six. The first group of six tubes serving as a control contained
medium A and larvae only. To each tube in groups 2, 3 and 9 were
added 50 µl of fresh untreated serum from mice given 2 immunising
doses of L_3, to groups 4 and 5, 50 µl of heat-inactivated serum from
these immunised mice, whilst to groups 6 and 7, 50 µl of fresh
untreated NMS. All tubes were incubated at 37° for 90 minutes.
After this period of incubation, 0.5 ml of medium B containing 2 x 10^6
peritoneal exudate cells from immunised mice were added to each tube
in groups 3, 5, 7 and 8. Group 8 served as a control containing
peritoneal exudate cells from immunised mice together with L_3 but no
serum. To groups 1, 2, 4 and 6 were added 0.5 ml of medium B.
Finally, to group 9, 2 x 10^6 peritoneal exudate cells from normal mice
were added in 0.5 ml of the same medium. All tubes were then incubated
at 37°C for 1 hour and the experimental procedure followed as outlined previously.

The results in Table 5.2 show that a significant reduction in the infectivity of L_3 occurred when the larvae were sensitised in the presence of either fresh NMS (0.01<p<0.05) or IMS(2) (p<0.01). However, incubation of exsheathed L_3 with these cells in the presence of heat-inactivated serum from immunised mice did not damage the L_3. The data show also that peritoneal exudate cells from normal mice in contrast to those from immune were ineffective in causing a reduction in larval infectivity even in the presence of fresh IMS(2).

These results together with those in Chapter 3 show that whilst antibody may mediate cell adherence it alone does not result in damage to the larvae. The effect of immune cells incubated with antibody and complement was significantly greater than that observed with immune cells and complement alone (0.01<p<0.05). Microscopic examination of the larvae showed that the cells adhering to their surface were predominantly macrophages.

5.3.3. COMPARISON BETWEEN THE EFFECT IN VITRO OF PERITONEAL EXUDATE CELLS FROM MICE GIVEN 2 IMMUNISING DOSES OF L_3 AND CELLS FROM MICE GIVEN 4 IMMUNISING DOSES OF L_3 ON THE INFECTIVITY OF EXSHEATHED L_3 SENSITISED WITH EITHER SERUM FROM MICE GIVEN 2 IMMUNISING DOSES OF L_3, IMS(2); OR SERUM FROM MICE GIVEN 4 IMMUNISING DOSES OF L_3, IMS(4).

The above results indicated that peritoneal exudate cells from mice infected with 2 doses of 200 L_3 were able to damage exsheathed L_3 in vitro leading to a reduction in their infectivity, providing these
TABLE 5.2

The effect in vitro of peritoneal exudate cells from mice given two immunising doses of $L_3$ on the infectivity of exsheathed $L_3$ sensitised with NMS or IMS(2) treated or untreated at 56°.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Sensitising serum</th>
<th>$L_3$ incubated with</th>
<th>Number of cysts*&lt;sub&gt;(X±SE, n=6)&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>no cells</td>
<td>144.5±4.2</td>
</tr>
<tr>
<td>2</td>
<td>IMS(2), unheated&lt;sup&gt;Δ&lt;/sup&gt;</td>
<td>no cells</td>
<td>136.7±4.5</td>
</tr>
<tr>
<td>3</td>
<td>IMS(2), unheated</td>
<td>immune cells&lt;sup&gt;▼&lt;/sup&gt;</td>
<td>93.5±10.0**</td>
</tr>
<tr>
<td>4</td>
<td>IMS(2) heat-inactivated</td>
<td>no cells</td>
<td>137.5±8.3</td>
</tr>
<tr>
<td>5</td>
<td>IMS(2) heat-inactivated</td>
<td>immune cells</td>
<td>140.0±6.4</td>
</tr>
<tr>
<td>6</td>
<td>NMS, unheated</td>
<td>no cells</td>
<td>134.7±7.2</td>
</tr>
<tr>
<td>7</td>
<td>NMS, unheated</td>
<td>immune cells</td>
<td>124.8±6.2*</td>
</tr>
<tr>
<td>8</td>
<td>None</td>
<td>immune cells</td>
<td>148.3±6.4</td>
</tr>
<tr>
<td>9</td>
<td>IMS(2), unheated</td>
<td>normal cells</td>
<td>140.2±6.1</td>
</tr>
</tbody>
</table>

<sup>Δ</sup> Serum was obtained from mice bled on day 11 after the final of 2 doses of 200 $L_3$ of N. dubius given orally 14 days apart. The serum was used either fresh or heat-inactivated at 56° for 30 minutes.

<sup>▼</sup> Peritoneal exudate cells were obtained from the same group of mice bled for serum.

# Analysed by Kruskall-Wallis one-way analysis of variance, p<.05; then analysed further by Mann-Whitney U test.

1 vs 2, 4, 5, 6, 8 or 9 not significant
1 vs 3  p = .002
1 vs 7  p = .034
3 vs 7  p = .016
3 vs 9  p = .006

* significant at $\alpha = .05$
** significant at $\alpha = .01$
larvae had been previously sensitised with serum from normal mice or mice given 2 immunising doses of $L_3$. Many workers have shown that the degree of resistance to this parasite is related to the number of immunising doses given (Van Zandt, 1961; Panter, 1967, 1969; Bartlett et al., 1972; Chaiumpya et al., 1977a). In view of this it was of some interest to know whether this was a reflection of a change in the activity of the cellular arm or a change in the humoral arm of defense.

Peritoneal exudate cells from mice infected orally with either 2 or 4 doses of 200 $L_3$ were collected and resuspended in medium B at the concentration of $4 \times 10^6$ cells/ml. Sera from these 2 groups of mice were freshly collected on the day of the experiment and kept on ice until required. Tubes containing 200 exsheathed $L_3$ in 0.2 ml of medium A were prepared and divided into 8 groups of 6. Group 1 serving as a control contained larvae in medium A only. To each tube in groups 2, 4 and 6 were added 50 µl of fresh untreated IMS(2), to groups 7 and 8, 50 µl of fresh untreated IMS(4) whilst to groups 3 and 5 serving as controls, 50 µl of medium A was added. All tubes were incubated at 37° for 90 minutes. After this incubation period, 0.5 ml of medium B containing $2 \times 10^6$ peritoneal exudate cells from mice infected with 2 doses of 200 $L_3$ were added to each tube in groups 3 and 4, whilst to groups 5, 6 and 8, 0.5 ml of medium B containing $2 \times 10^6$ peritoneal exudate cells from mice infected with 4 doses of 200$L_3$. To groups 2 and 7 containing no cells but serving as serum controls were also added 0.5 ml of medium B. The experimental protocol then followed the steps described previously.

The results presented in Table 5.3 show that irrespective of the number of immunising doses of $L_3$ that the mice had received, the cells were as efficient in damaging the larvae in the presence of IMS but
Comparison between the effect in vitro of peritoneal exudate cells from mice infected with 2 doses and 4 doses of 200 L₃, on the infectivity of exsheathed L₃ in the presence of IMS after 48 hour incubation at 37°C.

TABLE 5.3

<table>
<thead>
<tr>
<th>Group No.</th>
<th>L₃ incubated in the presence of</th>
<th>Number of cysts# (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum fromΔ mice</td>
<td>Peritoneal exudateΔ cells from mice</td>
</tr>
<tr>
<td>1</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>2</td>
<td>immunised twice</td>
<td>none</td>
</tr>
<tr>
<td>3</td>
<td>none</td>
<td>immunised twice</td>
</tr>
<tr>
<td>4</td>
<td>immunised twice</td>
<td>immunised twice</td>
</tr>
<tr>
<td>5</td>
<td>none</td>
<td>immunised x4</td>
</tr>
<tr>
<td>6</td>
<td>immunised twice</td>
<td>immunised x4</td>
</tr>
<tr>
<td>7</td>
<td>immunised x4</td>
<td>none</td>
</tr>
<tr>
<td>8</td>
<td>immunised x4</td>
<td>immunised x4</td>
</tr>
</tbody>
</table>

Serum and peritoneal exudate cells were obtained from mice on day 9 after the final of 2 or 4 doses of 200 L₃ of N. dubius given orally 14 days apart.

# Analyzed by Kruskall-Wallis one-way analysis of variance, p<.001; then analyzed further by Mann-Whitney U test

1 vs 2, 3 or 5 not significant
1 vs 4, 6, 7 or 8 p = .002
2 vs 7 p = .002
4 vs 6 vs 8 not significant
7 vs 8 not significant

** significant at α = .01
completely ineffective in its absence. One of the most surprising findings was that serum alone from mice that had received four immunising doses of L₃ killed the parasite whilst that from twice immunised animals did not. This property will be investigated further in Chapters 6 and 7.

5.3.4. THE EFFECT IN VITRO OF PERITONEAL EXUDATE CELLS FROM MICE GIVEN LIVING SALMONELLA ENTERITIDIS 11 RX ON THE INFECTIVITY OF EXSHEATHED L₃

The above results showed clearly that incubation in vitro of L₃ with peritoneal exudate cells from normal mice even in the presence of fresh IMS(2) did not affect the infectivity of the larvae. In contrast, incubation of L₃ with peritoneal exudate cells from immune mice with either fresh unheated NMS or IMS(2) could cause larval damage leading to the reduction in the infectivity of the larvae, suggesting that these cells were "activated" as had been previously reported (Chaicumpa et al., 1978). If this were so, it was of interest to determine whether cells activated by other means could function in a comparable manner to those mentioned above, providing the conditions of in vitro culture were the same as those for cells from immune mice. In order to investigate this phenomenon, macrophages which had been "activated" using S. enteritidis 11 RX were used.

A group of mice were injected intraperitoneally with $5 \times 10^5$ live S. enteritidis 11 RX which had been prepared as described in Materials and Methods. Eight days after this challenge the cells were harvested from the peritoneal cavity, washed and finally resuspended in medium B at a concentration of $4 \times 10^6$ cells/mL. Tubes containing 200 exsheathed L₃ in 0.2 mL of medium A were divided into nine groups of six. The first group serving as a control
contained medium A and larvae only. To each tube in groups 2, 3 and 9 were added 50 μl of fresh unheated IMS(2), to groups 4 and 5, 50 μl of heated IMS(2), whilst to groups 6 and 7, 50 μl of fresh unheated NMS. All tubes were incubated at 37°C for 90 minutes. At the end of this period, 0.5 ml of medium B containing $2 \times 10^6$ peritoneal exudate cells from mice infected with S. enteritidis 11 RX were added to each tube in groups 3, 5, 7 and 8. Group 8 served as a cell control containing peritoneal exudate cells from mice infected with S. enteritidis 11 RX incubated with L3 but no serum. To groups 1, 2, 4 and 6 were added 0.5 ml of medium B. Finally, to group 9 were added 0.5 ml of medium B containing $2 \times 10^6$ peritoneal exudate cells from mice immunised orally with 2 doses of 200 L3. This group served as a further control since these cells should have an effect upon the infectivity of the larvae. All tubes were then incubated at 37°C for 1 hour. After this period of incubation 5 ml of medium B were added to all tubes. After gassing with 5% CO2/air mixture, the tubes were then incubated for a further 48 hours at 37°C and the reduction in the infectivity determined as described previously.

The results in Table 5.4 show that a significant reduction in the infectivity of L3 occurred when the larvae were incubated with peritoneal exudate cells from mice infected with S. enteritidis 11 RX in the presence of fresh untreated serum from mice immunised twice against N. dubius or NMS $(.01<p<.05)$. The effect of these cells in the presence of both sources of serum was not significantly different. In contrast, these cells had no effect on larval infectivity in the presence of heated serum from twice immunised mice against N. dubius. The data show also that the effect of peritoneal exudate cells from mice infected with S. enteritidis 11 RX on the infectivity of L3 in the presence of fresh IMS(2) did not differ significantly from that
TABLE 5.4

Infectivity of exsheathed L₃ sensitised with either NMS or serum from mice given 2 immunising doses of N. dubius and incubated in vitro at 37° for 48 hours with peritoneal exudate cells from mice infected with S. enteritidis 11 RX.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Sensitising serum</th>
<th>Peritoneal exudate cells from mice infected with</th>
<th>Number of cysts# (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>none</td>
<td>nothing</td>
<td>143.7±9.5</td>
</tr>
<tr>
<td>2</td>
<td>IMS(2)Δ unheated</td>
<td>nothing</td>
<td>148.0±10.3</td>
</tr>
<tr>
<td>3</td>
<td>IMS(2) unheated</td>
<td>S. enteritidis 11 RX</td>
<td>109.2±9.8*</td>
</tr>
<tr>
<td>4</td>
<td>IMS(2) heat-inactivated</td>
<td>nothing</td>
<td>164.2±9.8</td>
</tr>
<tr>
<td>5</td>
<td>IMS(2) heat-inactivated</td>
<td>S. enteritidis 11 RX</td>
<td>153.3±4.9</td>
</tr>
<tr>
<td>6</td>
<td>NMS unheated</td>
<td>nothing</td>
<td>141.7±10.7</td>
</tr>
<tr>
<td>7</td>
<td>NMS unheated</td>
<td>S. enteritidis 11 RX</td>
<td>111.5±10.8*</td>
</tr>
<tr>
<td>8</td>
<td>none</td>
<td>S. enteritidis 11 RX</td>
<td>152.0±10.9</td>
</tr>
<tr>
<td>9</td>
<td>IMS(2) unheated</td>
<td>N. dubius</td>
<td>108.3±9.0*</td>
</tr>
</tbody>
</table>

Δ Serum was obtained from mice bled on day 10 after the final of 2 doses of 200 L₃ of N. dubius given intravenously 14 days apart. The serum was used either fresh or heat-inactivated at 56° for 30 minutes.

# Analysed by Kruskall-Wallis one-way analysis of variance, p<.01; then analysed further by Mann-Whitney U test

1 vs 2 vs 4 vs 6 not significant
1 vs 8 not significant
1 vs 3 p = .042
1 vs 7 p = .05
1 vs 9 p = .026
3 vs 7 or 9 not significant

* significant at α = .05
observed with cells from these same immunised mice.

These findings indicate that peritoneal exudate cells from S. enteritidis 11 RX stimulated mice were able to damage L₃ in vitro leading to the reduction in their infectivity but only in the presence of specific antibody and complement or complement alone. In contrast, these cells had no effect in vitro on the infectivity of L₃ in the presence of specific antibody alone.

5.4. CONCLUSION

Data presented in this chapter show that peritoneal exudate cells from mice infected with 2 doses of 200 L₃ N. dubius are capable of damaging L₃ in vitro, providing the larvae had been sensitised with antibody and complement or complement alone. However, they had no effect on the larvae if the parasite had been sensitised with antibody alone even though it mediated cell adherence. In addition, the effect of peritoneal exudate cells from mice infected with 4 immunising doses was no greater than that observed with cells from mice given 2 immunising doses. In contrast, peritoneal exudate cells from normal mice were unable to damage larvae even in the presence of both antibody and complement. This suggests that the cells from immunised mice which were predominantly macrophages were activated. This suggestion was supported by the results obtained from experiments in which the macrophages had been activated using S. enteritidis 11 RX. Peritoneal exudate cells from such stimulated mice were unable to damage larvae in the presence of antibody alone, but were able to do so in the presence of antibody and complement or complement alone.

The results also show that larval damage leading to a reduction in their infectivity can be achieved in vitro by fresh serum from mice infected with 4 doses of 200 L₃ N. dubius. This effect was not apparent when fresh serum from mice given 2 immunising doses was used.
CHAPTER 6

The effect of serum on the infectivity of $L_3$ following incubation in vitro.
6.1. **INTRODUCTION**

The work described in Chapter 5 showed that incubating *in vitro* exsheathed L₃ of *N. dubius* with serum freshly collected from mice previously infected with two doses of 200 L₃ had no effect on their infectivity. This was in agreement with results previously published by other workers (Chaucumpa et al., 1978; Prowse et al., 1978b). In contrast, incubation of L₃ with serum freshly collected from mice infected with four immunising doses resulted in larval damage as measured by a reduction in their infectivity. It seemed possible that as a result of several infections a larvicidal factor(s) was produced by the mouse and this may play a role in the development of host resistance. Therefore, it was of interest to study *in vitro* in more detail the effect of such serum on the larvae.

6.2. **METHOD**

**IN VITRO ASSAY FOR THE EFFECT OF A CELL-FREE SERUM SYSTEM ON THE INFECTIVITY OF EXSHEATHED L₃**

The assay was carried out in sterile 15 x 100 mm round bottomed siliconised glass tubes with a screw cap. Exsheathed L₃ washed twice in medium A and suspended at a concentration of 1000/ml were used. To each tube were added 0.2 ml of the larval suspension, 0.1 ml of various dilutions of serum freshly collected from immune or normal mice and 0.2 ml of supplemented medium A to give a final volume of 0.5 ml/tube. The tubes were then incubated at 37°C for 24 hours, after which time the contents were carefully removed and fed to mice, the contents of one tube being fed to one mouse. The control group contained larvae in supplemented medium A only. The number of cysts in the wall of the small intestine of each mouse was counted 6 days later. The results are expressed as the mean number of cysts in the 6 mice.
6.3. RESULTS

6.3.1. THE OPTIMAL INCUBATION TIME REQUIRED FOR SERUM FROM MICE INFECTED WITH 4 IMMUNISING DOES TO DAMAGE L₃

Evidence for the effect of serum from mice infected with 4 immunising doses to reduce the infectivity of L₃ was obtained from experiments investigating the interaction of peritoneal exudate cells with the parasite. In these experiments the larvae were incubated in the presence of IMS for a period of 48 hours. However it was possible that the time of incubation required for serum to produce an effect upon the infectivity of L₃ was much less. In view of this, the following experiments were carried out to determine the optimal incubation time.

Tubes containing 200 exsheathed L₃ in 0.2 ml of medium A were prepared and divided into three groups of thirty. To the first group of thirty tubes serving as a control, were added 0.3 ml of supplemented medium A only. To each tube in group 2 were added 0.1 ml of IMS(4) plus 0.2 ml of supplemented medium A so that the final dilution of serum was 1:5. To each tube in group 3 were added 0.1 ml of NMS plus 0.2 ml of supplemented medium A. All tubes were then incubated at 37°C for 0, 6, 24, 48, and 72 hours respectively. At the end of each incubation period, six tubes from each group were taken and the contents of each tube were fed to three groups of normal mice representing the three groups of tubes above. The mice were killed 6 days after challenge and the number of cysts in the wall of the small intestine counted.

The results in Table 6.1 show that no significant loss of infectivity had occurred after 6 hours of incubation of exsheathed L₃ with IMS(4), but significant loss had occurred after 24 hours incubation.
TABLE 6.1.
The effect of serum from either mice immunised orally with 4 doses of 200 L₃ or from normal mice on the infectivity of exsheathed L₃ of N. dubius following incubation in vitro for various times.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>L₃ incubated with</th>
<th>Time of incubation (hours)</th>
<th>Number of cysts† (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>1</td>
<td>no serum</td>
<td>163.7±6.2</td>
<td>166.5±9.5</td>
</tr>
<tr>
<td>2</td>
<td>IMS(4)* unheated</td>
<td>164.3±5.4</td>
<td>146.8±12.5</td>
</tr>
<tr>
<td>3</td>
<td>NMSΔ unheated</td>
<td>169.8±7.1</td>
<td>150.8±12.9</td>
</tr>
</tbody>
</table>

# Serum was freshly collected from mice bled on day 14 after the final of 4 oral doses of 200 L₃ of N. dubius given 14 days apart.

Δ Serum was freshly collected from normal mice.

† At 0 and 6 hours incubation, analysed by Kruskall-Wallis one-way analysis of variance 1 vs 2 vs 3, not significant

‡ At 24, 48 and 72 hours incubation, analysed by Kruskall-Wallis one-way analysis of variance, p<.001; then analysed further by Mann-Whitney U test 1 vs 2, p = .001
1 vs 3, not significant

** significant at α = .01
There was no further loss of infectivity over the next 24 hours.
However, after 72 hours of incubation it was found that the infectivity of exsheathed \( L_3 \) in all three groups decreased. Incubation of exsheathed \( L_3 \) with NMS for 48 hours did not reduce their infectivity. This experiment was repeated several times with similar results. In future experiments 24 hours was used as the standard incubation time.

6.3.2. LENGTH OF TIME REQUIRED FOR IMS(4) TO IMPAIR THE INFECTIONS OF \( L_3 \)

The previous experiment showed that IMS(4) was able to impair the infectivity of exsheathed \( L_3 \) following incubation of the larvae with IMS(4) in vitro for 24 hours but not after incubation for 6 hours. It was possible that the potential larvicidal reaction took place much more rapidly than this but it required 24 hours of incubation before it manifested itself.

To investigate this possibility experiments were carried out as follows. Tubes containing 200 exsheathed \( L_3 \) in 0.2 ml of medium A were divided into 6 groups of 6. Groups 1 and 2 serving as controls contained exsheathed \( L_3 \) only plus supplemented medium A. During the 24 hours incubation at 37° the larvae in the control group 2 were washed twice with 5 ml of medium A after 6 hours of incubation whilst group 1 served as an un-washed control. To each tube in groups 3, 4, 5 and 6 were added 50 \( \mu \)l of IMS(4) plus 0.25 ml of supplemented medium A to give a final dilution of serum of 1:10. All tubes were incubated at 37°. After one hour of incubation all tubes in group 3 were removed and the larvae washed twice with 5 ml of medium A, then resuspended in approximately 0.5 ml of this medium and incubated further. Groups 4 and 5 were treated in the same manner after 2 and 4 hours incubation respectively. Group 6, in which the larvae
were not washed, served as a serum positive control. All groups
were incubated for a total of 24 hours. At the end of the incubation
period, the contents of the tubes were fed to mice, one tube to one
mouse, and the reduction in the infectivity of exsheathed L₃ was
determined. The results are given in Table 6.2.

The data show that exsheathed L₃ which had been exposed to
IMS(4) for only one hour, washed, then incubated further in medium A
for up to 24 hours were impaired in their infectivity to the same
extent as if they had been in contact with IMS(4) for 24 hours.

6.3.3. THE LARVICIDAL ACTIVITY OF IMS(4) ON VARYING
NUMBERS OF EXSHEATHED L₃

Previous experiments have demonstrated that approximately 50%
of exsheathed L₃ which had been incubated with IMS(4) were damaged
as measured by a loss of infectivity. In these experiments 200
exsheathed L₃ were used and it seemed possible that if smaller
numbers of larvae were employed in these experiments the loss of
infectivity might be greater than that measured previously.

Tubes were divided into 4 groups of 12. To each tube in
group 1 were added 0.05 ml of a suspension of exsheathed L₃ suspended
in medium A at a concentration of 1,000/ml; whilst to tubes in groups
2, 3 and 4 were added 0.1 ml, 0.2 ml and 0.4 ml of the suspension.
To six tubes within each group were added 50 µl of IMS(4) plus
supplemented medium A to give a final volume in each tube of 0.5 ml.
To a further 6 tubes in each group serving as controls were added
supplemented medium A to give a final volume of 0.5 ml. All tubes
were then incubated at 37° for 24 hours and the reduction in the
infectivity of exsheathed L₃ determined as before. The results of
this experiment are shown in Table 6.3.
TABLE 6.2.
Length of time required for IMS(4) to impair the infectivity of L₃.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>L₃ exposed to</th>
<th>No. of cysts # (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>supplemented medium A</td>
<td>171.8±12.6</td>
</tr>
<tr>
<td>2</td>
<td>supplemented medium A (washed)</td>
<td>148.8±12.6</td>
</tr>
<tr>
<td>3</td>
<td>IMS(4) for 1 hour</td>
<td>66.5±9.4 **</td>
</tr>
<tr>
<td>4</td>
<td>IMS(4) for 2 hours</td>
<td>87.7±10.5 **</td>
</tr>
<tr>
<td>5</td>
<td>IMS(4) for 4 hours</td>
<td>71.0±7.4 **</td>
</tr>
<tr>
<td>6</td>
<td>IMS(4) for 24 hours</td>
<td>91.3±7.9 **</td>
</tr>
</tbody>
</table>

Δ Serum obtained from mice bled on day 9 after the final of 4 oral doses of 200 N. dubius L₃ given 14 days apart.

# Analysed by Kruskall-Wallis one-way analysis of variance, p<.001; then analysed further by Mann-Whitney U test

- 1 vs 2 not significant
- 1 vs 6 p = .002
- 2 vs 3, 4 or 5 p = .002
- 3 vs 6 not significant

** significant at α = .01
### TABLE 6.3.

The effect of a 1:10 dilution of IMS(4) on the infectivity of varying numbers of exsheathed L₃.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>No. of exsheathed L₃ used</th>
<th>L₃ incubated in 0.5 ml of medium A</th>
<th>1:10 serumΔ</th>
<th>No. of cysts (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>47.7±4.1</td>
<td>28.3±4.9</td>
<td>(59.4±10.3)</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>93.7±5.7</td>
<td>46.3±6.6</td>
<td>(45.9±9.9)</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>206.7±9.1</td>
<td>96.0±9.5</td>
<td>(46.4±4.6)</td>
</tr>
<tr>
<td>4</td>
<td>400</td>
<td>445.0±19.2</td>
<td>221.2±13.9</td>
<td>(49.7±3.1)</td>
</tr>
</tbody>
</table>

Δ Serum obtained from mice bled on day 10 after the final of 4 oral doses of 200 N. dubius L₃ given 14 days apart.

( ) percentage of cysts relative to controls ± standard error. The data were analysed by Kruskall-Wallis one-way analysis of variance.

1 vs 2 vs 3 vs 4, not significant.
It was found that when 50, 100, 200 and 400 exsheathed L₃ were incubated with 0.5 ml of 1:10 diluted IMS(4), the infectivity of larvae was still reduced at approximately 50% relative to their controls.

6.3.4. THE EFFECT OF THE FURTHER ADDITION OF IMS(4) DURING THE COURSE OF INCUBATION ON THE INFECTIVITY OF EXSHEATHED L₃

The data from the previous experiment suggested that the larvae may vary in their susceptibility to the larvicidal factor(s) in the IMS(4). In order to investigate this possibility the following experiment was carried out.

Tubes containing 200 exsheathed L₃ in 0.2 ml of medium A were divided into 4 groups of 6. Groups 1 and 4 serving as controls contained exsheathed L₃ in supplemented medium A only. To the tubes in groups 2 and 3 were added 50 µl of IMS(4) plus 0.25 ml of supplemented medium A to give a final concentration of serum of 1:10. All tubes in the 4 groups were incubated at 37°C for 24 hours. At times of 2 and 4 hours during this incubation period, the tubes in group 3 were taken and the larvae washed twice with 5 ml of medium A to remove excess serum, then supplemented with 50 µl of fresh serum from the same source at the end of each washing. Group 4, the control group, was treated in the same manner as group 3 but no serum was added at the end of each washing. At the end of 24 hours incubation, the contents of each tube were fed to an individual mouse. The reduction in the infectivity of exsheathed L₃ was determined by cysts counts 6 days later. The results are shown in Table 6.4.

The second experiment consisted of 3 groups of 6 tubes. Group 1 serving as a control contained exsheathed L₃ in supplemented medium A
only. To the tubes in groups 2 and 3 were added 50 μl of IMS(4) plus 0.25 ml of supplemented medium A. All tubes were incubated at 37° for 24 hours. At times of 2 and 4 hours during the incubation period, the tubes in group 3 were supplemented with 50 μl of fresh serum from the same source. At the end of 24 hours incubation period, the contents of each tube were fed to mice, one tube to one mouse. The reduction in the infectivity of exsheathed L₃ was determined 6 days later.

The results in Table 6.4 show that in both experiments, the addition of fresh IMS(4) during the incubation period did not further increase the number of larvae damaged.

6.3.5. **TITRATION OF THE LARVICIDAL ACTIVITY OF SERUM TAKEN AT VARIOUS TIMES DURING THE COURSE OF INFECTION FROM MICE INFECTED WITH VARYING NUMBER OF DOSES OF 200 N. DUBIUS L₃**

Mice were divided into 4 groups of 10. Mice in group 1 were infected orally with one dose of 200 N. dubius L₃; those in group 2 with 2 doses; group 3, 3 doses; and finally group 4, 4 doses. The doses were given at 14 day intervals. All mice in the 4 groups were bled 10 days after the last dose and groups 1, 2 and 3 once again on day 50 after the primary dose. Sera freshly collected from these mice were titrated for their ability to damage exsheathed L₃ when incubated with them *in vitro*, as follows.

Tubes containing 200 exsheathed L₃ in 0.2 ml of medium A were prepared and divided into 6 groups of 6. Group 1 serving as a control contained exsheathed L₃ and supplemented medium A only. To each tube in groups 2 to 6, 0.1 ml of either undiluted or diluted serum plus 0.2 ml of supplemented medium A were added to give a final dilution of
The Table 6.4.
The effect of the further addition of IMS(4) during the course of incubation on the infectivity of exsheathed $L_3$.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Exsheathed $L_3$ in the presence of</th>
<th>Experiment I ($\bar{x}$±SE, n=6)</th>
<th>Experiment II</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>medium A</td>
<td>176.3±7.7</td>
<td>205.8±5.4</td>
</tr>
<tr>
<td>2</td>
<td>1:10 IMS(4) with no serum supplement</td>
<td>104.2±11.3**</td>
<td>123.3±14.0**</td>
</tr>
<tr>
<td>3</td>
<td>1:10 IMS(4) with serum supplement</td>
<td>70.0±11.3**</td>
<td>108.5±14.6**</td>
</tr>
<tr>
<td>4</td>
<td>medium A (washing†)</td>
<td>172.0±11.4</td>
<td>—</td>
</tr>
</tbody>
</table>

$\Delta$ Exsheathed $L_3$ were incubated with serum from 4 times $N.$ dubius infected mice without supplementing with fresh serum during 24 hours incubation in vitro.

$\n$ In experiment I, exsheathed $L_3$ were incubated with IMS(4) for 24 hours in vitro. At times 2 and 4 hours during the incubation period the larvae were washed twice with 5 ml of medium A and supplemented with fresh serum from the same source. In experiment II, at times 2 and 4 hours of incubation an additional 50 µl of IMS(4) were added without washing the larvae.

† Exsheathed $L_3$ were incubated with medium A serving as a control and washed with medium A at times 2 and 4 hours of incubation.

#: Experiment I, analysed by Kruskall-Wallis one-way analysis of variance, $p<.001$; then analysed further by Mann-Whitney U test.

1 vs 4, not significant
1 vs 2, $p = .004$
3 vs 4, $p = .002$

#: Experiment II, analysed by Kruskall-Wallis one-way analysis of variance, $p<.001$; then analysed further by Mann-Whitney U test.

1 vs 2 or 3, $p = .002$
2 vs 3, not significant

** significant at $\alpha = .01$
serum of 1:5, 1:15, 1:45, 1:135 and 1:405 respectively. All tubes were incubated at 37° for 24 hours and the reduction in the infectivity of exsheathed L₃ determined as described previously.

The results of this experiment are shown in Table 6.5 and Table 6.6 where it may be seen that the titre of the larvicidal activity reached a maximum 10 days after a second dose of larvae. It is interesting to note that approximately 50 days after the primary dose irrespective of the number of immunising infections there was no significant difference in the titre between the various groups.

6.3.6. COMPARISON BETWEEN THE EFFECTS OF SERUM FROM MICE INFECTED WITH 4 DOSES OF N. DUBIUS L₃ AND SERUM FROM MICE INFECTED WITH MESOCESTOIDES CORTI ON THE INFECTIONITY OF EXSHEATHED L₃

Since in vitro incubation of exsheathed L₃ with IMS(4) resulted in a reduction of larval infectivity, it was important to know whether serum from mice infected with other parasitic helminths could similarly damage the larvae.

In order to determine this, mice infected originally with 100 tetrathyridia of Mesocestoides corti were used. M. corti is a cestode parasite which gives rise to a chronic infection in mice, the parasite multiplying in the liver and peritoneal cavity. The population of tetrathyridia in a mouse arising from a small inoculum of 10-100 parasites may reach proportions of several thousands without apparently affecting the health of the animal.

On the day of the experiment serum was collected from mice which had been infected with M. corti 7 months previously. Sera from normal mice and mice infected with 4 doses of 200 N. dubius L₃
# TABLE 6.5.

Titration of the larvicidal activity of serum from mice infected with various doses of 200 *N. dubius* L$_3$ taken on day 10 after the final dose.

<table>
<thead>
<tr>
<th>No. of doses given 200 L$_3$/dose</th>
<th>Time of bleeding after the last dose of L$_3$</th>
<th>Medium A</th>
<th>Dilution of serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:5</td>
<td>1:15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:5</td>
<td>1:15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:5</td>
<td>1:15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:5</td>
<td>1:15</td>
</tr>
<tr>
<td>1</td>
<td>day 10</td>
<td>144.8±3.6</td>
<td>101.5±9.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:5</td>
<td>1:15</td>
</tr>
<tr>
<td>2</td>
<td>day 10</td>
<td>144.8±3.6</td>
<td>91.8±7.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:5</td>
<td>1:15</td>
</tr>
<tr>
<td>3</td>
<td>day 10</td>
<td>144.8±3.6</td>
<td>114.3±9.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:5</td>
<td>1:15</td>
</tr>
<tr>
<td>4</td>
<td>day 10</td>
<td>162.5±19.9</td>
<td>109.7±11.2</td>
</tr>
</tbody>
</table>

N.D. not done

( ) $\bar{x}$±SE of percentage cysts relative to control.
TABLE 6.6.

Titration of the larvicidal activity of serum from mice infected with various doses of 200 *N. dubius* L<sub>3</sub> taken on day 50 after the first dose.

<table>
<thead>
<tr>
<th>No. of doses given 200 L&lt;sub&gt;3&lt;/sub&gt;/dose</th>
<th>Time of bleeding after the first dose of L&lt;sub&gt;3&lt;/sub&gt;</th>
<th>Medium A</th>
<th>Dilution of serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:5</td>
<td>1:15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:135</td>
<td>1:45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No. of cysts ((\bar{x} \pm SE), n=6)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>day 50</td>
<td>162.5±19.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>82.3±11.0</td>
<td>98.3±14.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(50.7±6.8)</td>
<td>(60.5±8.8)</td>
</tr>
<tr>
<td>2</td>
<td>day 50</td>
<td>162.5±19.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>95.7±9.3</td>
<td>103.7±11.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(58.9±5.8)</td>
<td>(63.8±6.8)</td>
</tr>
<tr>
<td>3</td>
<td>day 50</td>
<td>162.5±19.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>81.8±14.9</td>
<td>71.0±10.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(50.4±9.1)</td>
<td>(43.7±6.3)</td>
</tr>
</tbody>
</table>

N.D. not done

( ) \(\bar{x} \pm SE\) of percentage cysts relative to control
were also prepared. All sera were used before and after heat-inactivation to destroy complement. Tubes containing 200 exsheathed L₃ in 0.2 ml of medium A were prepared and divided into 10 groups of 6. Group 1, the control group, contained larvae and supplemented medium A but no serum. To each tube in groups 2 and 3, 0.1 ml of either fresh undiluted or diluted IMS(4) and 0.2 ml of supplemented medium A were added to make a final dilution of serum of 1:5 and 1:20 respectively. To each tube in groups 4 and 5, 0.1 ml of heat-inactivated IMS(4) was added. In addition, to group 4 were added 0.2 ml of supplemented medium A and group 5 was supplemented with 0.1 ml of fresh NMS plus 0.1 ml of supplemented medium A. Groups 6, 7, 8 and 9 were set up in a similar manner to those of groups 2, 3, 4 and 5 respectively but serum from M. corti infected mice was used. Finally, to each tube in group 10 were added 0.1 ml of fresh NMS plus 0.2 ml of supplemented medium A. All tubes were incubated at 37° for 24 hours and the infectivity of the larvae determined as previously described.

The results in Table 6.7 show that following incubation of exsheathed L₃ with unheated IMS(4), there was a reduction in the infectivity of the L₃, when compared with the infectivity of the larvae in the control group (p<0.01). In contrast, incubation of exsheathed L₃ with heat-inactivated IMS(4) did not affect the infectivity of the larvae, and the activity could not be restored by adding fresh NMS as a source of complement.

The inability of NMS to restore activity to heat-inactivated IMS(4) might have been due to complement consumption by aggregated immunoglobulins in the heated serum. However, when one volume of the above heat-inactivated IMS(4) had been incubated with an equal
TABLE 6.7.
Comparison between the effect of serum from mice infected with 4 doses of *N. dubius* L₃ and serum from mice infected with *M. corti* on the infectivity of exsheathed L₃.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>L₃ incubated in the presence of serum from</th>
<th>Dilution of serum</th>
<th>No. of cysts (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>175.3±7.3</td>
</tr>
<tr>
<td>2</td>
<td>N. dubius, unheated</td>
<td>1:5</td>
<td>88.8±5.6**</td>
</tr>
<tr>
<td>3</td>
<td>N. dubius, unheated</td>
<td>1:20</td>
<td>102.0±6.7**</td>
</tr>
<tr>
<td>4</td>
<td>N. dubius, heated</td>
<td>1:5</td>
<td>167.7±5.9</td>
</tr>
<tr>
<td>5</td>
<td>N. dubius, heated &amp; NMS</td>
<td>1:5</td>
<td>152.7±6.4</td>
</tr>
<tr>
<td>6</td>
<td>M. corti, unheated</td>
<td>1:5</td>
<td>156.2±5.8</td>
</tr>
<tr>
<td>7</td>
<td>M. corti, unheated</td>
<td>1:20</td>
<td>164.2±11.7</td>
</tr>
<tr>
<td>8</td>
<td>M. corti, heated</td>
<td>1:5</td>
<td>189.3±3.7</td>
</tr>
<tr>
<td>9</td>
<td>M. corti, heated &amp; NMS</td>
<td>1:5</td>
<td>182.5±7.7</td>
</tr>
<tr>
<td>10</td>
<td>NMS, unheated</td>
<td>1:5</td>
<td>158.5±6.7</td>
</tr>
</tbody>
</table>

† Serum was freshly collected from mice bled on day 10 after the final of 4 oral doses of 200 L₃ of *N. dubius* given 14 days apart.

▼ Serum was freshly collected from mice infected 7 months previously with 100 *M. corti*.

△ Serum was freshly collected from normal mice.

# Analysed by Kruskall-Wallis one-way analysis of variance, p<.001, then analysed further by Mann-Whitney U test.

1 vs 4, 5, 6, 7, 8, 9 or 10, not significant
1 vs 2 or 3  

** significant at α = .01
volume of fresh NMS at $37^\circ$ for 2 hours prior to determine hemolytic complement activity as described in Materials and Methods, it was found that this serum had the hemolytic complement activity of 2.30 CH$_{50}$ units, compared with fresh unheated IMS(4) of 2.46 CH$_{50}$ units. This result shows clearly that there was no difference in hemolytic complement activity in these two serum samples. Thus the inability of NMS to restore the activity to heat inactivated IMS(4) was unlikely to have been due to complement consumption by aggregated immunoglobulins. The data also show that incubation of exsheathed L$_3$ with fresh unheated serum from mice infected with *M. corti* an unrelated helminth parasite did not reduce their ability to infect normal mice, indicating a degree of specificity in the reaction.

### 6.3.7. THE EFFECT OF STORING IMS(4) ON ICE FOR VARYING PERIODS OF TIME ON ITS LARVICIDAL ACTIVITY

IMS(4) was collected and divided into four 2.25 ml aliquots and kept on ice until use. At times 0, 2, 5 and 7 days after storage on ice, an aliquot of serum was taken and titrated for its ability to damage exsheathed L$_3$. For each time point, tubes containing 200 exsheathed L$_3$ in 0.2 ml of medium A were prepared and divided into 8 groups of 6. Group 1, the control group, contained exsheathed L$_3$ and supplemented medium A only. To group 2 through to group 8 were added 0.1 ml of either undiluted or diluted IMS(4) plus 0.2 ml of supplemented medium A such that the final dilution of serum was 1:5, 1:20, 1:50, 1:100, 1:200, 1:400 and 1:800 respectively. All tubes were then incubated at $37^\circ$ for 24 hours and the reduction in the infectivity of exsheathed L$_3$ determined as described previously.

The results in Table 6.8 show that IMS(4) retained its larvicidal activity for a period of two days whilst being stored on ice.
TABLE 6.8.

The effect of storing IMS(4) on ice for varying periods of time in its larvicidal activity.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Exsheathed L₃ incubated in the presence of</th>
<th>Time of storage (days)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No. of cysts# (X±SE, n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>supplemented medium A</td>
<td>169.2±4.3</td>
<td>150.8±6.1</td>
<td>156.8±11.9</td>
<td>165.5±9.7</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>IMS(4)Δ diluted 1:5</td>
<td>101.5±3.6**</td>
<td>96.3±3.6**</td>
<td>127.7±9.5*</td>
<td>138.8±14.6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>IMS(4) diluted 1:20</td>
<td>92.5±3.6**</td>
<td>97.3±3.9**</td>
<td>131.8±11.4</td>
<td>165.2±5.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>IMS(4) diluted 1:50</td>
<td>106.5±3.2**</td>
<td>84.3±2.6**</td>
<td>138.2±8.4</td>
<td>159.0±11.4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>IMS(4) diluted 1:100</td>
<td>103.3±2.4**</td>
<td>126.8±3.9</td>
<td>148.7±13.4</td>
<td>177.5±8.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>IMS(4) diluted 1:200</td>
<td>131.8±1.9</td>
<td>151.3±4.3</td>
<td>188.8±9.8</td>
<td>177.8±6.8</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>IMS(4) diluted 1:400</td>
<td>155.7±4.5</td>
<td>165.3±3.2</td>
<td>194.7±8.8</td>
<td>174.3±6.3</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>IMS(4) diluted 1:800</td>
<td>178.2±2.1</td>
<td>169.8±3.0</td>
<td>189.3±6.7</td>
<td>180.3±6.5</td>
<td></td>
</tr>
</tbody>
</table>

Δ Serum was obtained from mice bled on day 9 after the final of 4 oral doses of 200 N. dubius L₃ given 14 days apart.

The serum was divided into 4 aliquots of 2.25 ml each and kept on ice for a period of 0, 2, 5 and 7 days respectively. At each time point, one aliquot was taken and assayed for its ability to damage exsheathed L₃ following incubation in vitro.

# Analyzed by Kruskall-Wallis one-way analysis of variance, then analyzed further by Mann-Whitney U test

:At 0 days

1 vs 2, 3, 4 or 5, p = .002
1 vs 6, 7 or 8, not significant

:At 2 days

1 vs 2, 3, or 4, p = .008
1 vs 5, 6, 7 or 8, not significant

:At 5 days

1 vs 2, p = .042
1 vs 3, 4, 5, 6, 7 or 8, not significant

:At 7 days (analyzed only by Kruskall-Wallis one-way analysis of variance)

1 vs 2, 3, 4, 5, 6, 7 or 8, not significant

** significant at α = .01
* significant at α = .05
Storage for longer periods of time resulted in a loss of activity.

6.3.8. **THE EFFECT OF STORING IMS(4) AT -20° ON ITS ABILITY TO DAMAGE L₃ IN VITRO**

It was of interest to determine whether IMS(4) which had been kept at -20° for a period of time still retained the ability to damage exsheathed L₃.

The experiment was carried out as follows. IMS(4) was collected and kept frozen at -20°. Two months later, the serum was thawed and an aliquot heat-inactivated at 56°. Tubes containing 200 exsheathed L₃ in 0.2 ml of medium A were prepared and divided into 4 groups of 6. Group 1, the control group, contained the larvae plus supplemented medium A. To each tube in group 2 was added 0.1 ml of unheated IMS(4); to tubes in group 3, 0.1 ml of heat-inactivated IMS(4) and to tubes in group 4, 0.1 ml of fresh NMS. Finally, to each tube in all the groups, were added 0.2 ml of supplemented medium A to give a final concentration of serum of 1:5. The tubes were then incubated at 37° for 24 hours and the reduction in the infectivity of the larvae determined.

The results in Table 6.9 show that IMS(4) which had been kept frozen at -20° for 2 months had lost its larvicidal activity.

6.3.9. **THE EFFECT OF HEAT TREATMENT AT DIFFERENT TEMPERATURES ON THE LARVICIDAL ACTIVITY OF IMS(4)**

IMS(4) was collected and divided into three aliquots of 0.8 ml each. One of the three aliquots was kept on ice as a serum positive control. The remaining two aliquots of serum were treated by heating at 45° for 30 minutes and 56° for 30 minutes respectively. All these three serum samples were then titrated for their ability to damage exsheathed L₃ as follows.
TABLE 6.9.

The effect of storing IMS(4) at -20°C on its ability to damage L₃ in vitro.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Exsheathed L₃ incubated in†</th>
<th>No. of cysts# (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>supplemented medium A</td>
<td>156.0±8.3</td>
</tr>
<tr>
<td>2</td>
<td>untreated IMS(4)Δ, stored at -20°C</td>
<td>167.0±6.3</td>
</tr>
<tr>
<td>3</td>
<td>heat-inactivated IMS(4), after storage at -20°C</td>
<td>157.2±7.6</td>
</tr>
<tr>
<td>4</td>
<td>fresh NMS</td>
<td>162.8±5.6</td>
</tr>
</tbody>
</table>

† The final dilution of serum in each group was 1:5

Δ Serum was obtained from mice bled on day 10 after the final of 4 oral doses of 200 N. dubius L₃ given 14 days apart, and kept frozen at -20°C for 2 months. The serum prior to being frozen for 2 months reduced the infectivity of L₃ by 61.5±2.3% relative to the control.

# Analysed by Kruskall-Wallis one-way analysis of variance 1 vs 2 vs 3 vs 4, not significant.
Tubes containing 200 exsheathed L3 in 0.2 ml of medium A were set up and divided for each serum into 3 groups of 6. A control group 4 contained larvae and supplemented medium A only. To each tube in group 1, 0.1 ml of 1:2 diluted serum was added, to group 2, 0.1 ml of 1:4 diluted serum and to group 3, 0.1 ml of 1:8 diluted serum. Finally, 0.2 ml of supplemented medium A were added to each tube in groups 1, 2 and 3 to give a final dilution of serum of 1:10, 1:20 and 1:40 respectively. All these tubes were then incubated at 37° for 24 hours and the reduction in the infectivity of exsheathed L3 determined. The results are shown in Table 6.10.

The data show clearly that the larvicidal activity of serum was stable to heating at 45° for 30 minutes, but was completely destroyed by heating at 56° for a similar length of time.

6.3.10. THE EFFECT OF DIALYSIS ON THE LARVICIDAL ACTIVITY OF IMS(4)

Serum from immune mice was freshly collected and divided into 2 aliquots of 2 ml each. One of these aliquots was kept on ice overnight and served as a serum positive control. The remaining aliquot of serum was transferred to a dialysis bag and dialysed at 4° overnight against two changes of 1 litre of pre-cooled 10 mM HEPES-buffered saline containing 14 mM Ca++ and 8 mM Mg++. Both non-dialysed and dialysed serum were then titrated for their ability to damage exsheathed L3 as follows.

Tubes containing 200 exsheathed L3 in 0.2 ml of medium A were divided into 6 groups of 6. Group 1 contained exsheathed L3 and supplemented medium A only serving as a control. To each tube in groups 2, 3, 4, 5 and 6, 0.1 ml of undiluted or diluted serum plus 0.2 ml of supplemented medium A were added to give a final
TABLE 6.10.
The ability of IMS(4) to damage exsheathed $L_3$ in vitro after heat treatment.

<table>
<thead>
<tr>
<th>Treatment of sera†</th>
<th>Dilution of serum</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:10</td>
<td>1:20</td>
<td>1:40</td>
<td></td>
</tr>
<tr>
<td>Heated 56°/30 min</td>
<td></td>
<td>181.0±11.6</td>
<td>177.2±7.8</td>
<td>178.8±6.6</td>
</tr>
<tr>
<td>Heated 45°/30 min</td>
<td></td>
<td>68.7±11.9**</td>
<td>82.8±10.3**</td>
<td>88.2±5.1**</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>71.3±7.2**</td>
<td>72.5±11.8**</td>
<td>67.5±9.6**</td>
</tr>
</tbody>
</table>

Control: Group 4 exsheathed $L_3$ + medium A only 157.0±8.8.

† Serum from mice bled on day 14 after the final of 4 oral doses of 200 N. dubius $L_3$ given 14 days apart.

# Analysed by Kruskall-Wallis one-way analysis of variance, p<.001; then analysed further by Mann-Whitney U test.

- Heated $56^\circ$ /30 min.: 4 vs 1, 2 or 3, not significant
- Heated $45^\circ$ /30 min.: 4 vs 1, 2 or 3, $p = .002$
- None: 4 vs 1, 2 or 3, $p = .002$

** significant at $\alpha = .01$
dilution of serum of 1:5, 1:20, 1:50, 1:100 and 1:200 respectively. All these tubes were then incubated at 37°C for 24 hours and the reduction in the infectivity of exsheathed L₃ was determined. The results are shown in Figure 6.1. The data show that the larvicidal activity of the serum was not lost on dialysis.

6.3.11. **THE REQUIREMENT FOR COMPLEMENT IN MEDIATING DAMAGE TO EXSHEATHED L₃ IN VITRO BY SERUM FROM MICE INFECTED WITH 4 IMMUNISING DOSES OF N. DUBIUS L₃**

The above results showed that following incubation of L₃ with serum from mice infected with 4 immunising doses of N. dubius there was a significant loss of infectivity. The larvicidal activity was destroyed by heating and could not be restored by the addition of NMS as a source of complement.

In order to investigate further whether larval damage required the presence of complement, fresh unheated IMS(4) was passed through an anti-mouse C3 column to remove the C3 component of complement as described in Materials and Methods. Both fresh IMS(4) and C3 depleted serum were then tested for their ability to reduce the infectivity of L₃. Tubes containing 200 exsheathed L₃ in medium A were prepared and divided into 15 groups of 6. Group 1 serving as controls, contained larvae and supplemented medium A only. To each tube in groups 2 to 8, 0.1 ml of either fresh undiluted or diluted IMS(4) and 0.2 ml of supplemented medium A were added such that the final dilutions of serum were of 1:5, 1:20, 1:50, 1:100, 1:200, 1:400 and 1:800 respectively. The other remaining 7 groups of 6 tubes were set up in a similar fashion to those of groups 2 to 8, but C3 depleted serum was used instead of fresh IMS(4). All tubes
FIGURE 6.1.
The ability of dialysed and non-dialysed serum from 4 times *N. dubius* infected mice to damage exsheathed $L_3$ following incubation *in vitro*. Vertical bars represent mean ± standard error of the number of cysts counted on day 6.
DIALYSED SERUM
NON-DIALYSED SERUM
MEDIUM A CONTROL

NUMBER OF CYSTS COUNTED

DILUTION OF SERUM

MEDIUM A CONTROL
were incubated at 37\(^\circ\) for 24 hours and the infectivity of the larvae determined as described previously.

The results in Table 6.11 show that incubation of L\(_3\) with fresh unheated IMS(4) resulted in a reduction in the infectivity of the L\(_3\). In contrast, incubation of L\(_3\) with IMS(4) that had been passed through an anti-mouse C3 column to remove the C3 component of complement did not affect the infectivity of the larvae. It seemed therefore, from this evidence that complement was required in mediating larval damage \textit{in vitro} by IMS(4).

6.3.12. \textbf{THE ABILITY OF IMS(4) AFTER REMOVAL OF THE IMMUNOGLOBULINS TO DAMAGE EXSHEATHED L\(_3\)}

The previous data showed that complement appeared to be required in the reaction that led to larval damage when exsheathed L\(_3\) were incubated together with IMS(4) at 37\(^\circ\). However it was clear that complement alone had no effect, even though the larvae are known to activate complement by the alternate pathway (Prowse \textit{et al.}, 1979b) since NMS did not impair the ability of L\(_3\) to infect normal mice.

In view of this it was important to know whether specific antibodies or some other factor(s) in IMS(4) participated with complement in mediating the damage. To clarify this situation the following experiments were carried out.

IMS(4) freshly collected was passed down an anti-mouse F(ab')\(_2\) column to remove the immunoglobulins as described in Materials and Methods. In addition an aliquot of the IMS(4) was passed down an anti-mouse C3 column to remove the third component of complement. These adsorbed sera together with unadsorbed serum were then tested for their ability to reduce the infectivity of L\(_3\). Tubes containing 200 exsheathed L\(_3\) in 0.2 ml of medium A were prepared and divided into 4 groups of 6. Group 1 serving as a control contained larvae
TABLE 6.11.

Infectivity of L3 incubated in vitro with either fresh IMS(4) or IMS(4) after removal of the C3 component of complement.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Dilution of serum incubated with L3</th>
<th>Fresh IMS(4)</th>
<th>C3 negative IMS(4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of cysts† (X±SE, n=6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>no serum</td>
<td>155.3±8.1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1:5</td>
<td>78.5±5.9**</td>
<td>169.0±6.2</td>
</tr>
<tr>
<td>3</td>
<td>1:20</td>
<td>73.3±10.5**</td>
<td>169.2±11.3</td>
</tr>
<tr>
<td>4</td>
<td>1:50</td>
<td>86.0±10.8**</td>
<td>159.2±3.3</td>
</tr>
<tr>
<td>5</td>
<td>1:100</td>
<td>79.5±6.9**</td>
<td>173.8±9.1</td>
</tr>
<tr>
<td>6</td>
<td>1:200</td>
<td>109.8±12.6*</td>
<td>168.2±14.9</td>
</tr>
<tr>
<td>7</td>
<td>1:400</td>
<td>133.0±6.9</td>
<td>163.5±4.9</td>
</tr>
<tr>
<td>8</td>
<td>1:800</td>
<td>168.5±7.5</td>
<td>160.0±6.3</td>
</tr>
</tbody>
</table>

Δ Serum was freshly collected from mice bled on day 10 after the final of 4 oral doses of 200 L3 of N. dubius given 14 days apart.

† Analysed by Kruskall-Wallis one-way analysis of variance, p<.001, then analysed further by Mann-Whitney U test.

** significant at α = .01
* significant at α = .05
and supplemented medium A only. To each tube in group 2 were added 50 µl of untreated IMS(4), to group 3, 50 µl of IMS(4) after passage through an anti-mouse C3 column, whilst to group 4, 50 µl of IMS(4) chromatogrammed on an anti-mouse F(ab')2 column. Finally to each tube in groups 2, 3 and 4 were added 0.25 ml of supplemented medium A to give a final dilution of serum of 1:10. All tubes were incubated at 37°C for 24 hours and the reduction in the infectivity determined as described previously.

The results are shown in Table 6.12. The data indicate that IMS(4) from which the immunoglobulins had been removed still reduced the infectivity of L₃ when incubated with them in vitro, and was as active in this regard as the original IMS(4). However, from this result it was not possible to conclude that specific antibodies were not involved in mediating larval damage. Since, by an ELISA assay (see Materials and Methods) it was found that approximately 10% of the immunoglobulins still remained in the serum after passage down an anti-mouse F(ab')₂ column. Although the immunoglobulin level in adsorbed serum was low, this might have been sufficient to produce an effect upon larval infectivity. The data also show that removal of C3 from IMS(4) completely inactivates its larvicidal properties.

6.4. CONCLUSIONS

The data in this chapter show that incubation in vitro of exsheathed L₃ with IMS(4) for a period of 24 hours resulted in larval damage as measured by a reduction in their infectivity. Further it was found that larvae which had been exposed to IMS(4) for only one hour, washed and then incubated further in supplemented medium A for up to 24 hours were impaired in their infectivity to the same extent as if they had been in contact with IMS(4) for
TABLE 6.12
The effect of IMS(4), IMS(4) from which either the immunoglobulins or the third component of complement has been removed on the infectivity of L₃.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>L₃ incubated with</th>
<th>Number of cysts# (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>supplemented medium A</td>
<td>183.2±4.4</td>
</tr>
<tr>
<td>2</td>
<td>fresh untreated IMS(4)✓</td>
<td>94.3±9.3**</td>
</tr>
<tr>
<td>3</td>
<td>IMS(4) minus C3†</td>
<td>161.2±7.8**</td>
</tr>
<tr>
<td>4</td>
<td>IMS(4) minus Igθ</td>
<td>83.3±6.6**</td>
</tr>
</tbody>
</table>

✓ Serum was freshly collected from mice bled on day 10 after the final of 4 oral doses of 200 N. dubius L₃ given 14 days apart.

† The above serum after removal of the component C3 by chromatography on an anti-mouse C3 affinity column.

θ The above serum after removal of immunoglobulins by chromatography on an anti-mouse F(ab')₂ affinity column.

△ The final dilution of serum was 1:10.

# Analysed by Kruskall-Wallis one-way analysis of variance, p<.001; then analysed further by Mann-Whitney U test.

1 vs 2 or 4 p = .002
1 vs 3 not significant
2 vs 4 not significant

** significant at α = .01
24 hours. Only approximately 50% of the larvae appeared to be sensitive to the larvicidal activity of IMS(4) and further addition of IMS(4) to larvae during the incubation period did not increase the loss of infectivity compared with those that had been incubated with a finite amount. Studies of the larvicidal activity of serum taken at various times during the course of infection from mice infected with varying number of doses of 200 N. dubius L₃ showed that the titre of the larvicidal activity reached a maximum 10 days after a second dose of L₃ and approximately 50 days after the primary dose irrespective of the number of immunising infections. There was no significant difference in the titre between the various groups.

The data show also that incubation of exsheathed L₃ with fresh unheated serum from mice infected with M. corti an unrelated helminth parasite did not reduce their ability to infect normal mice which indicated a degree of specificity in the reaction. The data show further that the larvicidal activity of IMS(4) was heat labile, but the activity could not be restored by the addition of fresh NMS as a source of complement.

It was found that IMS(4) stored on ice retained its larvicidal activity for a period of two days but storage for longer periods of time resulted in a complete loss of activity as did storage at -20°C for 2 months. The larvicidal activity of serum was stable to heating at 45°C for 30 minutes, but was completely destroyed by heating at 56°C for a similar length of time. The larvicidal activity of serum was not lost on dialysis.

Incubation of L₃ in fresh unheated IMS(4) resulted in a reduction in the infectivity of the L₃ whereas incubation of L₃ with IMS(4) that had been passed through an anti-mouse C3 column to remove the C3 component of complement led to a complete loss of
activity. It seemed therefore that complement was required in mediating larval damage. The data indicate also that IMS(4) from which the major proportion of the immunoglobulins had been removed still reduced the infectivity of L3 when incubated with them in vitro. Although the immunoglobulin level in the serum was low, this might have been sufficient to produce an effect on the larvae.
CHAPTER 7

Protection afforded to normal mice to *N. dubius* infection following the passive transfer of serum from mice resistant to re-infection.
7.1. INTRODUCTION

The work described in the previous chapter has shown that incubating exsheathed \( L_3 \) with immune serum freshly collected from mice that had been infected orally on 4 occasions with *N. dubius* resulted in a reduction in their infectivity. At least one of the components involved in the larvicidal activity of the IMS appeared to be complement.

Generally speaking it has not been possible to transfer immunity passively by serum taken from immune mice with one notable exception (see literary review). However in view of the larvicidal activity of serum from multiply infected mice it seemed important to establish whether such serum could protect naive mice against this parasite.

7.2. RESULTS

7.2.1. PASSIVE TRANSFER OF IMMUNITY TO NAIVE MICE GIVEN IMS(4) INTRAPERITONEALLY.

Mice were divided into 2 groups of 6 animals. Mice in group 1 were injected intraperitoneally with 0.5 ml of IMS(4) whilst those in group 2 were given 0.5 ml of saline. The animals were then challenged orally with 200 \( L_3 \). Twenty-four hours after challenge the mice were again given intraperitoneally a similar dose of serum which had been kept on ice overnight or saline.

Seven days after feeding the larvae all mice were killed and the number of cysts in the wall of the small intestine counted.

The data given in Table 7.1 show that mice injected intraperitoneally with IMS(4) were as susceptible to the infection as were the control group.
TABLE 7.1.
Passive transfer of immunity to naive mice given IMS(4) intraperitoneally.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Treatment of group</th>
<th>No. of cysts# (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IMS(4)Δ</td>
<td>172.8±10.5</td>
</tr>
<tr>
<td>2</td>
<td>saline (control)</td>
<td>181.7±10.5</td>
</tr>
</tbody>
</table>

Δ Serum obtained from mice bled on day 14 after the final of 4 oral doses of 200 L₃ given 14 days apart.

# Analysed by Mann-Whitney U test
   1 vs 2, not significant
7.2.2. PASSIVE TRANSFER OF IMMUNITY TO NAIVE MICE BY THE INTRAVENOUS INJECTION OF IMS(4)

The results of the previous experiment showed that mice injected intraperitoneally with IMS(4) were not protected against a subsequent oral challenge. It was possible that the route of administration of serum as well as the route of challenge may be important factors in the expression of resistance. The following experiments were carried out in order to clarify this situation.

Mice were divided into 3 groups of 6 animals. Mice in group 1 acting as controls were injected intravenously with 0.5 ml of saline. Mice in group 2 received 0.5 ml of IMS(4), whilst those in group 3 received serum from mice that had been given two oral doses of L₃, IMS(2). The mice within these 3 groups were then challenged intravenously with 200 exsheathed L₃ 10 minutes after the administration of serum or saline. Seven days after challenge all mice were killed and the number of cysts counted.

The data in Table 7.2 show that mice which had received IMS(4) were protected against a challenge infection of exsheathed L₃. In contrast, mice which had received IMS(2) were not protected.

7.2.3. PASSIVE TRANSFER OF IMMUNITY TO NAIVE MICE BY THE INTRAVENOUS INJECTION OF IMS(4) FOLLOWED BY A CHALLENGE INFECTION OF EITHER EXSHEATHED L₃ OR SHEATHED L₃.

Mice were divided into 7 groups of 6 animals. Groups 1 and 6 acting as controls were injected intravenously with 0.5 ml of saline. Mice in groups 2 and 7 received 0.5 ml of fresh untreated IMS(4). Those in group 3 were injected with IMS(4) which had been inactivated at 56°C for 30 minutes. Animals in group 4 were given fresh untreated
TABLE 7.2.

Passive transfer of immunity to *N. dubius* infection to naive mice by the intravenous injection of either IMS(4) or IMS(2).

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Treatment</th>
<th>No. of cysts# (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>saline (control)</td>
<td>140.0±10.7</td>
</tr>
<tr>
<td>2</td>
<td>IMS(4)</td>
<td>85.8±9.2**</td>
</tr>
<tr>
<td>3</td>
<td>IMS(2)</td>
<td>116.2±4.2</td>
</tr>
</tbody>
</table>

▼ Serum obtained from mice bled on day 10 after the final of 4 oral doses of 200 L₃ given 14 days apart.

▲ Serum obtained from mice bled on day 10 after the final of 2 oral doses of 200 L₃ given 14 days apart.

# Analyzed by Kruskall-Wallis one-way analysis of variance, p< .01; then analyzed further by Mann-Whitney U test.

1 vs 2, \( p = .008 \)
1 vs 3, not significant

** significant at \( \alpha = .01 \)
IMS(2) and those in group 5 received the same serum that had been heat-inactivated. Ten minutes after the administration of serum the mice in groups 1, 2, 3, 4 and 5 were challenged intravenously with 200 exsheathed L3, whilst those in groups 6 and 7 were injected with 200 sheathed L3. All mice were then killed 7 days after challenge and the number of cysts counted.

The data in Table 7.3 show that mice given fresh untreated IMS(4) were protected against a challenge infection of exsheathed L3. However when heat-inactivated IMS(4) or IMS(2) either fresh or heat-inactivated was given to recipient mice there was no significant protection. Likewise mice given fresh untreated IMS(4) were not protected against subsequent challenge with sheathed L3.

The interesting finding resulting from this experiment was that IMS(4) heated at 56°C for 30 minutes was unable to protect naive mice when given passively. This was in agreement with the results given in Chapter 6 where it was shown that in vitro the larvicidal activity of heated IMS(4) could not be re-established by the addition of fresh IMS as a complement source. This suggests that the other factor(s) involved in the larvicidal activity were also heat labile.

7.2.4. THE ABILITY OF VARIOUS DILUTIONS OF IMS(4) TO TRANSFER IMMUNITY PASSIVELY TO NAIVE MICE.

The results of the previous experiments showed that fresh untreated IMS(4) was capable of transferring protection to naive mice, providing the mice were challenged intravenously. It was of interest to determine to what extent IMS(4) could be diluted before the protective effect was lost.

Mice were divided into 8 groups of 6 animals. Group 1 acting as controls received intravenously 0.25 ml of saline. Group 2
TABLE 7.3.

Mean cyst recovery from 6 mice given intravenously either IMS(4) or IMS(2) 10 minutes prior to intravenous challenge with 200 exsheathed L₃ or 200 sheathed L₃.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Challenge infection</th>
<th>0.5 ml of serum transferred</th>
<th>No. of cysts (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Exsheathed L₃</td>
<td>saline (control)</td>
<td>114.0±6.6</td>
</tr>
<tr>
<td>2</td>
<td>Exsheathed L₃</td>
<td>IMS(4)</td>
<td>65.2±7.5**</td>
</tr>
<tr>
<td>3</td>
<td>Exsheathed L₃</td>
<td>heat-inactivated IMS(4)</td>
<td>103.2±9.9</td>
</tr>
<tr>
<td>4</td>
<td>Exsheathed L₃</td>
<td>IMS(2)</td>
<td>90.7±7.1</td>
</tr>
<tr>
<td>5</td>
<td>Exsheathed L₃</td>
<td>heat-inactivated IMS(2)</td>
<td>110.2±7.1</td>
</tr>
<tr>
<td>6</td>
<td>Sheathed L₃</td>
<td>saline (control)</td>
<td>105.8±6.2</td>
</tr>
<tr>
<td>7</td>
<td>Sheathed L₃</td>
<td>IMS(4)</td>
<td>89.5±4.9</td>
</tr>
</tbody>
</table>

▼ Serum obtained from mice bled on day 10 after the final of 4 oral doses of 200 L₃ given 14 days apart.

▲ Serum obtained from mice bled on day 10 after the final of 2 oral doses of 200 L₃ given 14 days apart.

# Analysed by Kruskall-Wallis one-way analysis of variance, p<.01; then analysed by Mann-Whitney U test.

1 vs 2,
1 vs 3, 4 or 5, not significant
6 vs 7, not significant

** significant at α = .01
received intravenously 0.5 ml of fresh untreated IMS(4), whilst group 3 were given 0.25 ml of the serum. Mice in groups 4, 5, 6, 7 and 8 were injected intravenously with 0.25 ml of IMS(4) diluted in saline to a concentration of 1:2, 1:4, 1:8, 1:16 and 1:32 respectively. All mice were then challenged intravenously with 200 exsheathed L3 10 minutes after the administration of the serum. Seven days after challenge all mice were killed and the number of cysts in the wall of the small intestine counted.

The results given in Table 7.4 show that significant protection against the parasite was achieved when as little as 0.25 ml of a 1:8 dilution of the serum was transferred to naive mice. If one assumes that the blood volume of a mouse is 10% of its body weight then this would represent a final dilution of approximately 1:80, since the mice used in this experiment were approximately 25 gms in weight.

7.2.5. THE EFFECT OF FREEZING AND THAWING ON THE ABILITY OF IMS(4) TO TRANSFER PROTECTION TO NAIVE MICE.

Previous experiments in vitro had shown that IMS(4) that had been kept at -20°C lost its larvicidal activity following thawing. Other experiments had indicated that one of the labile factors involved was complement since the removal of C3 from IMS(4) completely inactivated the in vitro larvicidal activity of the serum. Further evidence suggested that a second factor requiring the participation of complement was involved in the larvicidal activity of IMS(4). A likely candidate for this second factor was specific antibody although the in vivo experiment showing the lack of protection using heat inactivated IMS(4) mitigated against this possibility. However if it were specific antibody it should be stable to storage
TABLE 7.4.
Mean cyst recovery from 6 mice given intravenously various dilutions of IMS(4) 10 minutes prior to challenge with 200 exsheathed \( L_3 \).

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Mice received</th>
<th>No. of cysts(^#) (( \bar{X} \pm SE, n=6 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.25 ml saline (control)</td>
<td>123.3( \pm )6.2</td>
</tr>
<tr>
<td>2</td>
<td>0.5 ml IMS(4)( ^\Delta )</td>
<td>73.3( \pm )7.5 **</td>
</tr>
<tr>
<td>3</td>
<td>0.25 ml IMS(4)</td>
<td>68.8( \pm )5.3 **</td>
</tr>
<tr>
<td>4</td>
<td>0.25 ml 1:2 IMS(4)</td>
<td>86.7( \pm )7.2 **</td>
</tr>
<tr>
<td>5</td>
<td>0.25 ml 1:4 IMS(4)</td>
<td>98.8( \pm )6.0 **</td>
</tr>
<tr>
<td>6</td>
<td>0.25 ml 1:8 IMS(4)</td>
<td>75.8( \pm )3.9 **</td>
</tr>
<tr>
<td>7</td>
<td>0.25 ml 1:16 IMS(4)</td>
<td>101.5( \pm )10.4</td>
</tr>
<tr>
<td>8</td>
<td>0.25 ml 1:32 IMS(4)</td>
<td>101.0( \pm )5.9</td>
</tr>
</tbody>
</table>

\( ^\Delta \) Serum obtained from mice bled on day 16 after the final of 4 oral doses of 200 \( L_3 \) given 14 days apart.

\( ^\# \) Analysed by Kruskall-Wallis one-way analysis of variance, \( p<.001 \); then analysed further by Mann-Whitney U test

- 1 vs 2, \( p = .004 \)
- 1 vs 3 or 6, \( p = .002 \)
- 1 vs 4 or 5, \( p = .008 \)
- 1 vs 7 or 8, not significant
- 2 vs 6, not significant

** significant at \( \alpha = .01 \)
at -20°C and therefore such a stored serum should be capable of transferring immunity passively. Freshly collected IMS(4) was divided into 2 aliquots of 2.5 ml each. One aliquot of the serum was kept on ice overnight as serum positive control while the other was kept at -20°C. The following day the aliquot of serum was thawed and kept on ice until required.

Mice were divided into 3 groups of 6 animals. Group 1 acting as a control received intravenously 0.4 ml of saline. Group 2 were injected intravenously with 0.4 ml of IMS(4) that had been incubated on ice overnight, whilst group 3 received the serum that had been kept at -20°C for the same length of time. All mice were challenged intravenously with 200 exsheathed L₃ 10 minutes after the administration of the serum. Seven days after challenge, all mice were killed and the number of cysts in the small intestine counted.

The results in Table 7.5 demonstrated that IMS(4) that had been kept at -20°C was still able to transfer immunity passively to naive mice, as one might expect if the labile component was complement. This experiment was repeated with similar results.

7.2.6. COMPARISON BETWEEN THE IN VIVO AND IN VITRO LARVICIDAL EFFECT OF IMS(4) AFTER CHROMATOGRAPHY ON EITHER AN ANTI-MOUSE C3 OR AN ANTI-MOUSE F(ab')₂ COLUMN.

In vitro studies in the previous chapter showed that complement together with possibly specific antibodies were involved in damaging exsheathed L₃.

In order to study the relationship between complement and specific antibody in mediating damage to the parasite in more detail
TABLE 7.5.
The effect of freezing and thawing on the ability of IMS(4) to transfer protection to naive mice to N. dubius infection.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Mice received 0.4 ml of</th>
<th>No. of cysts# (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>saline</td>
<td>82.8±11.5</td>
</tr>
<tr>
<td>2</td>
<td>IMS(4)Δ kept at 0°</td>
<td>31.0±3.1**</td>
</tr>
<tr>
<td>3</td>
<td>IMS(4) kept at -20°</td>
<td>25.0±4.1**</td>
</tr>
</tbody>
</table>

Δ Serum obtained from mice bled 20 days after the final of 4 oral doses of 200 L₃ given 14 days apart.

# Analysed by Kruskall-Wallis one-way analysis of variance, p<.01; then analysed further by Mann-Whitney U test
1 vs 2 or 3, p = .002
2 vs 3, not significant

** significant at α = .01
the following in vitro and in vivo experiments were carried out.

A 15 ml aliquot of IMS(4) was collected and divided into aliquots of 4, 5 and 6 ml respectively. The 4 ml aliquot of serum served as an untreated serum control. The 5 ml aliquot of serum was passed down an anti-mouse C3 column to remove C3 (see section 2.22), the effluent obtained was 4.7 ml. In a similar manner, the 6 ml aliquot of serum was chromatographed on an anti-mouse F(ab')₂ column to remove the immunoglobulins (see section 2.21), the effluent obtained was 14 ml. Both effluents were then assayed in vitro for their larvicidal activity as follows.

Tubes containing 200 exsheathed L₃ in 0.2 ml of medium A were prepared and divided into 4 groups of 6. Tubes in group 1 contained exsheathed L₃ in supplemented medium A only. To those in group 2 were added 50 µl of untreated IMS(4); to each tube in group 3, 50 µl of the serum effluent from the anti-mouse C3 column; whilst tubes in group 4 received 100 µl of the serum effluent from the anti-mouse F(ab')₂ column. Supplemented medium A was then added to all tubes to give a final volume of 0.5 ml. This resulted in a final dilution of IMS(4) and the effluents from the columns of 1:10. The tubes were then incubated at 37°C for 24 hours and the reduction in the infectivity of the exsheathed L₃ was determined. The results are given in Table 7.6.

At the same time the IMS(4) and the column effluents were assayed for their ability to transfer immunity passively to naive mice. Mice were divided into 4 groups of 6 animals. Group 1 received intravenously 0.5 ml of saline, group 2 received a similar volume of untreated IMS(4), group 3 the effluent from the anti-mouse C3 column, whilst group 4 received the effluent from the anti-mouse F(ab')₂ column. Ten minutes later each mouse was challenged
intravenously with 200 exsheathed L₃ and the number of cysts counted on the 7th day after challenge. The results are given in Table 7.6 where it may be seen that removal of C3 completely destroys the larvicidal activity of IMS(4) in vitro, but as might be expected transfer of such depleted serum to mice still results in protection against an intravenous challenge. In contrast IMS(4) from which the immunoglobulins had been removed was completely ineffective in vivo in protecting the animal against N. dubius. The fact that this serum was active in vitro could be accounted for by the small amount of immunoglobulin still present in the effluent. Analysis by ELISA indicated that only 90% of the immunoglobulins had been removed following passage through the anti-F(ab')₂ column. The experiment was repeated with similar results.

7.2.7. FURTHER EVIDENCE THAT COMPLEMENT IS INVOLVED IN THE LARVICIDAL ACTIVITY OF IMS(4).

The previous in vitro and in vivo studies implicated the involvement of complement in the larvicidal activity of IMS(4). The following experiments were carried out to establish unequivocally that complement was a necessary component of the mechanism which resulted in larval damage. It has been reported that methylamine inactivates C3, C4 and α₂-macroglobulin (Pangburn et al., 1981) and further it has been suggested that this reagent is a milder way of inactivating complement than heat-treatment since this latter method may cause some aggregation of the immunoglobulins.

In initial experiments, a 2 ml aliquot of IMS(4) freshly collected was incubated with 0.2 ml of 1M methylamine-HCl/0.13 M sodium phosphate (pH 8.0) at room temperature for 1 hour, then kept on ice for 4 hours. The serum was then dialysed with stirring overnight against PBS and finally against 50 ml of medium A
TABLE 7.6.

Relationship between the *in vivo* and *in vitro* effect of IMS(4) after passed down either anti-mouse C3 affinity column or anti-mouse F(ab')₂ affinity column on the infectivity of exsheathed L₃.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>in vivo Δ</th>
<th>in vitro †</th>
<th>No. of cysts # (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>102.7±12.0</td>
<td>183.2±4.4</td>
<td></td>
</tr>
<tr>
<td>2 IMS(4) V</td>
<td>27.0±3.0 **</td>
<td>94.3±9.3 **</td>
<td></td>
</tr>
<tr>
<td>3 IMS(4) minus C3</td>
<td>53.3±16.3 *</td>
<td>161.2±7.8</td>
<td></td>
</tr>
<tr>
<td>4 IMS(4) minus Ig</td>
<td>95.2±15.4</td>
<td>83.3±6.6 **</td>
<td></td>
</tr>
</tbody>
</table>

V Serum obtained from mice bled on day 10 after the final of 4 oral doses of 200 L₃ given 14 days apart.

Δ Mice received intravenously 0.5 ml of saline or serum.

† The final dilution of serum in each tube was 1:10.

# Analysed by Kruskall-Wallis one-way analysis of variance, then analysed further by Mann-Whitney U test

<table>
<thead>
<tr>
<th></th>
<th>in vivo</th>
<th>in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 vs 2</td>
<td>p = .002</td>
<td>p = .002</td>
</tr>
<tr>
<td>1 vs 3</td>
<td>p = .042</td>
<td>not significant</td>
</tr>
<tr>
<td>1 vs 4</td>
<td>not significant</td>
<td>p = .002</td>
</tr>
</tbody>
</table>

* significant at α = .05

** significant at α = .01
for 1 hour. The methylamine treated serum, together with the untreated serum were then tested for their ability to damage exsheathed \( L_3 \) in vitro in the following manner.

Tubes containing 200 exsheathed \( L_3 \) in 0.2 ml of medium A were prepared and divided into 11 groups of 6. Group 1 serving as a control contained larvae in supplemented medium A only. To tubes in groups 2, 3 and 4 were added 0.1 ml of untreated pre-diluted IMS(4) plus 0.2 ml of supplemented medium A to give a final dilution of serum of 1:15, 1:45 and 1:135 respectively. To those in groups 5, 6 and 7 were added 0.1 ml of pre-diluted methylamine-treated serum plus 0.2 ml of supplemented medium A to give a final dilution of serum as above. To each tube in groups 8, 9 and 10 were added 0.1 ml of pre-diluted methylamine-treated serum, 0.1 ml of NMS diluted 1:6 and 0.1 ml of supplemented medium A to give a final dilution of the methylamine-treated serum of 1:15, 1:45 and 1:135 respectively. The final dilution of NMS in each tube was thus 1:30. Finally to tubes in group 11 serving as a control group were added 0.1 ml of NMS diluted 1:6 plus 0.2 ml of supplemented medium A to give a final dilution of serum of 1:30. All tubes were incubated at 37°C for 24 hours and the reduction in the infectivity determined as described previously.

The results in Table 7.7 show that treatment of IMS(4) with methylamine greatly reduced its larvicidal activity. This activity could be restored if NMS used as a source of complement were added to the treated serum. It is also clear that in this experiment complement was the limiting component in this reaction since untreated IMS(4) to which NMS had not been added was inactive at a dilution of 1:135 whereas the methylamine treated serum in the presence of NMS was still highly active at a similar dilution.
TABLE 7.7.

The effect of methylamine-treated IMS(4) in the presence or absence of NMS on the infectivity of L₃ following incubation in vitro.

<table>
<thead>
<tr>
<th>Exsheathed L₃ incubated in the presence of</th>
<th>Control</th>
<th>Dilution of serum</th>
<th>No. of cysts* (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>medium A</td>
<td>Gp. 1</td>
<td>1:15</td>
<td>1:45</td>
</tr>
<tr>
<td>IMS(4)</td>
<td>Gp. 2</td>
<td>73.2±7.8 **</td>
<td>96.0±6.8 *</td>
</tr>
<tr>
<td>IMS(4), i</td>
<td>Gp. 5</td>
<td>98.8±7.1 *</td>
<td>135.7±14.4</td>
</tr>
<tr>
<td>IMS(4), i + NMS(1/30)</td>
<td>Gp. 8</td>
<td>97.7±7.1 *</td>
<td>99.7±10.4 *</td>
</tr>
<tr>
<td>NMS(1/30)</td>
<td>Gp. 11</td>
<td>146.8±20.9</td>
<td></td>
</tr>
</tbody>
</table>

Δ Serum obtained from mice bled 9 days after the final of 4 oral doses of 200 L₃ given 14 days apart.

∇ Serum as above incubated with methylamine (100 mM) at room temperature for 1 hour and then kept on ice for 4 hours.

# Analyzed by Kruskall-Wallis one-way analysis of variance, p<.001; then analyzed further by Mann-Whitney U test

1 vs 2, p = .002
1 vs 3 or 5, p = .012
1 vs 8, p = .016
1 vs 9, p = .026
1 vs 10, p = .042
1 vs 4, 6, 7, or 11, not significant

* significant at α = .05
** significant at α = .01
7.2.8. THE EFFECT OF AN IMMUNOGLOBULIN PREPARATION OR AN IgG AND IgM FRACTION PREPARED FROM IMS(4) ON THE INFECTIVITY OF EXSHEATHED L₃ IN THE PRESENCE AND ABSENCE OF NMS.

Previous experiments had indicated that specific antibody was the second factor involved in the larvicidal activity of the IMS(4). In order to confirm this, IgG and IgM fractions were prepared from IMS(4) and assayed for their ability to damage exsheathed L₃ following incubation in vitro. The experiments were carried out as follows.

Serum was obtained from mice bled on day 25 after the final of 4 doses of 200 L₃ given orally 14 days apart. Immunoglobulins were prepared from a 10.5 ml aliquot of the serum by ammonium sulphate precipitation (see section 2.16). The precipitate was removed by centrifuging and redissolved in tris-buffered saline, pH 8.0/0.1 mM EDTA/8 mM NaN₃. The concentrated immunoglobulin preparation was found to contain 41.9 mg/ml of proteins as measured by reading OD at 230 nm, then divided into aliquots of 2.74 ml and 2.5 ml. The 2.74 ml aliquot was dialysed at 4°C for 16 hours against two changes of 500 ml of saline and finally against 4 changes of 1 litre batches of PBS, pH 7.4/0.15 mM CaCl₂/0.5 mM MgCl₂ for periods of 30 minutes and held on ice for three hours until required.

The 2.5 ml aliquot of the immunoglobulin preparation was applied to a Sephacryl S-200 column (see section 2.18). The IgM and IgG fractions were pooled separately and concentrated to the original volume (2.5 ml). These fractions were then dialysed at 4°C for 3 hours against 4 changes of 1 litre batches of PBS, pH 7.4/0.15 mM CaCl₂/0.5 mM MgCl₂. IgM fraction was found
to contain 4.9 mg/ml of proteins as measured by OD at 280 nm whilst IgG fraction contained 34.2 mg/ml of proteins. These fractions were then assayed for their larvicidal activity.

On the day of the experiment, two days after the initial bleeding to provide the immunoglobulin preparation, the same group of mice were bled. The serum was collected and divided into 2 aliquots of 1 ml each. One aliquot was held on ice until used in the assay as a positive serum control, the other was inactivated by methylamine treatment (see section 2.9).

Tubes containing 200 exsheathed L3 in 0.2 ml of medium A were prepared and divided into 11 groups of 6. Group 1 contained the larvae in supplemented medium A only serving as the negative control. To the tubes in groups 2 and 3 were added 50 µl of the immunoglobulin fraction obtained by ammonium sulphate precipitation. To those in groups 4 and 5 were added 50 µl of the IgM fraction while groups 6 and 7 received the same volume of the IgG fraction. To group 8 were added 50 µl of untreated IMS(4) and to groups 9 and 10, 50 µl of methylamine-treated IMS(4). In addition to groups 3, 5, 7, 10 and 11 was added 0.1 ml of fresh NMS. Finally to all tubes in each group was added supplemented medium A such that the final volume was 0.5 ml/tube. These tubes were then incubated at 37° for 24 hours and the reduction in the infectivity of the larvae determined.

The results in Table 7.8 show that immunoglobulin fraction prepared by ammonium sulphate precipitation, the IgM fraction, IgG fraction and methylamine-treated IMS(4) had no effect on the infectivity of the larvae when incubated with them in vitro. However with the exception of the IgG fraction the larvicidal activity of these fractions was restored by the addition of fresh NMS.
The effect of various immunoglobulin preparations and methylamine-treated serum in the presence or absence of NMS on the infectivity of exsheathed $L_3$ following incubation in vitro.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Exsheathed $L_3$ in the presence of</th>
<th>No. of cysts ($\bar{x}\pm SE$, n=6) $^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>medium A (control)</td>
<td>$181.3\pm9.4$</td>
</tr>
<tr>
<td>2</td>
<td>Ig $^\Delta$</td>
<td>$189.2\pm10.4$</td>
</tr>
<tr>
<td>3</td>
<td>Ig + NMS</td>
<td>$139.8\pm11.6^*$</td>
</tr>
<tr>
<td>4</td>
<td>IgM $^#$</td>
<td>$215.7\pm9.7$</td>
</tr>
<tr>
<td>5</td>
<td>IgM + NMS</td>
<td>$118.3\pm6.9^{**}$</td>
</tr>
<tr>
<td>6</td>
<td>IgG $^#$</td>
<td>$205.5\pm10.3$</td>
</tr>
<tr>
<td>7</td>
<td>IgG + NMS</td>
<td>$153.8\pm22.6$</td>
</tr>
<tr>
<td>8</td>
<td>IMS $^\nabla$</td>
<td>$137.3\pm6.0^{**}$</td>
</tr>
<tr>
<td>9</td>
<td>methylamine-treated IMS</td>
<td>$207.0\pm7.3$</td>
</tr>
<tr>
<td>10</td>
<td>methylamine-treated IMS + NMS</td>
<td>$105.0\pm6.9^{**}$</td>
</tr>
<tr>
<td>11</td>
<td>NMS</td>
<td>$177.3\pm7.3$</td>
</tr>
</tbody>
</table>

$^\nabla$ Serum obtained from mice bled on day 25 after the final of 4 oral doses of 200 $L_3$ given 14 days apart.

$^\Delta$ Immunoglobulin fraction prepared by ammonium sulphate precipitation.

$^#$ IgM and IgG fractions prepared from IMS(4) by fractionation on Sephacryl S-200.

$^+$ Analysed by Kruskall-Wallis one-way analysis of variance, $p<.001$; then analysed further by Mann-Whitney U test.

1 vs 2, 4, 6, 7, 9 or 11, not significant
1 vs 3, $p = .03$
1 vs 5, $p = .004$
1 vs 8, $p = .008$
1 vs 10, $p = .002$
8 vs 3, 5 or 10, not significant

* significant at $\alpha = .05$
** significant at $\alpha = .01$
The results indicate that specific antibodies together with complement are important factors involved in the killing of exsheathed L₃ \textit{in vitro}. Although, IgG and IgM fractions prepared by the method indicated above were contaminated with other serum proteins, the IgM was more effective in the killing of the larvae than IgG providing complement was present.

7.2.9. \textbf{THE EFFECT OF PURIFIED IgM PREPARED FROM IMS(4) ON THE INFECTIVITY OF EXSHEATHED L₃ \textbf{IN THE PRESENCE OF NMS.}}

The above results showed that the IgM fraction prepared from IMS(4) had an effect upon the infectivity of exsheathed L₃ providing complement was present, whereas the IgG fraction in the presence of complement did not. Since the IgM fraction used in the previous experiment was contaminated with serum proteins other than IgM, it was possible that the contaminating protein(s) were responsible for the larvicidal activity of this particular fraction in the presence of complement. The following experiments were carried out to clarify this situation.

Serum was obtained from mice bled on day 20 after the final of 4 oral doses of 200 L₃ given 14 days apart. An aliquot of the serum (29.4 ml) was inactivated with methylamine by the method described in section 2.9. The immunoglobulins were prepared by ammonium sulphate precipitation of this inactivated serum (see section 2.16). The precipitate was removed by centrifuging and redissolved in 18 ml of tris-buffered saline, pH 8.0/0.1 mM EDTA/8 mM NaN₃. The concentrated immunoglobulin preparation was found to contain 100 mg/ml of IgG and 0.48 mg/ml of IgM as measured by ELISA. Mouse IgM was then purified by passing a 15 ml aliquot
of the immunoglobulin preparation down a Sephacryl S-300 column followed by DEAE-Sephacel (see section 2.18). The IgM fraction was assayed by ELISA and found to contain 0.3 mg/ml of IgM and 12.2 μg/ml of IgG. The purified IgM was dialysed against 2 litres of saline before being assayed for its larvicidal activity.

On the day of the experiment, a group of mice previously injected with 4 oral doses of 200 L₃ were bled 20 days after the last dose. The serum was collected and held on ice until used in the assay as a positive serum control.

Tubes containing 200 exsheathed L₃ in 0.2 ml of medium A were prepared and divided into 8 groups of 6. Group 1 containing the larvae in supplemented medium A only served as the negative control. To the tubes in group 2 were added 100 μl of fresh NMS(4), to groups 3 and 4 100 μl of pure IgM whilst to groups 5, 6 and 7 50 μl, 25 μl and 12.5 μl of pure IgM respectively. In addition to groups 4, 5, 6, 7 and 8 was added 0.1 ml of fresh NMS. Finally to all tubes in each group were added supplemented medium A such that the final volume was 0.5 ml/tube. At this time the final concentration of IgM in groups 3, 4, 5, 6 and 7 were 60, 60, 30, 15 and 7.5 μg/ml respectively. All tubes were then incubated at 37°C for 24 hours and the reduction in the infectivity of the larvae determined.

The results in Table 7.9 show that pure IgM in the presence of fresh NMS reduced the infectivity of exsheathed L₃ when incubated with them in vitro, but had no effect in the absence of NMS. The larvicidal activity of IgM in the presence of fresh NMS was apparent even at a concentration of as small as 7.5 μg/ml.

The data show clearly that pure IgM together with complement are important factors involved in the killing of exsheathed L₃ in vitro.
TABLE 7.9.
The effect of pure IgM in the presence or absence of NMS on the infectivity of L₃ following incubation in vitro.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>L₃ incubated with</th>
<th>No. of cysts* (X±SE, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>supplemented medium A</td>
<td>168.7±8.0</td>
</tr>
<tr>
<td>2</td>
<td>1:5 IMS(4)^Δ</td>
<td>114.5±13.5**</td>
</tr>
<tr>
<td>3</td>
<td>60 µg/ml IgM^v</td>
<td>187.7±11.8</td>
</tr>
<tr>
<td>4</td>
<td>60 µg/ml IgM + 1:30 NMS</td>
<td>109.3±4.9**</td>
</tr>
<tr>
<td>5</td>
<td>30 µg/ml IgM + 1:30 NMS</td>
<td>104.0±12.7**</td>
</tr>
<tr>
<td>6</td>
<td>15 µg/ml IgM + 1:30 NMS</td>
<td>110.2±14.7**</td>
</tr>
<tr>
<td>7</td>
<td>7.5 µg/ml IgM + 1:30 NMS</td>
<td>127.0±12.7*</td>
</tr>
<tr>
<td>8</td>
<td>1:30 NMS</td>
<td>154.5±15.9</td>
</tr>
</tbody>
</table>

^Δ Serum obtained from mice bled on day 20 after the final of 4 oral doses of 200 L₃ given 14 days apart.

^v Pure IgM was prepared from IMS(4) by chromatography on Sephacyl S-300, followed by DEAE-Sephacel.

# Analyzed by Kruskall-Wallis one-way analysis of variance, p<.001; then analyzed further by Mann-Whitney U test.

1 vs 3 or 8, not significant
1 vs 2, p = .004
1 vs 4, p = .002
1 vs 5, p = .006
1 vs 6, p = .012
1 vs 7, p = .042
7.3. CONCLUSIONS

The data in this chapter show that fresh IMS(4) given intravenously to naive mice protects them against a subsequent intravenous challenge with exsheathed L₃, but not against a challenge with sheathed L₃. The protective effect of IMS(4) was abolished by heat treatment but not by freezing/thawing.

IMS(4) from which the C3 component had been removed had in vitro lost its larvicidal activity but was fully active in vivo passive transfer experiments. In contrast the same serum from which the greater proportion of the immunoglobulins had been removed was unable to transfer passively to naive mice resistance to infection with *N. dubius*. Further experiments indicated that the larvicidal activity of serum from mice that had received four infections of *N. dubius* was dependent on specific antibody and complement, the antibody being of the IgM class and not IgG.
CHAPTER 8

Discussion
DISCUSSION

The present study has examined in detail the interaction between mouse peritoneal macrophages with various developmental forms of *N. dubius* and the ability of IMS to damage exsheathed L₃ in the absence of phagocytic cells. Initial experiments were carried out to study the adherence of peritoneal exudate cells to the surface of the parasite and the factors involved in promoting this adhesion. The data showed clearly that in the absence of serum peritoneal exudate cells from normal mice did not adhere to the cuticle of the exsheathed L₃. However, exposure of the larvae to NMS in VBS i.e. in the presence of Ca⁺⁺ and Mg⁺⁺ promoted strong adherence of normal peritoneal exudate cells to the cuticle. This adhesion was abolished if the larvae were sensitised with NMS in the presence of EDTA but not if the larvae were sensitised with the serum in the presence of EGTA. This indicated that the cells were binding to the larvae via their C3 receptors as a result of attachment of C3b to the cuticle of the larvae and confirms previous observations by Prowse et al., (1979b) that this parasite may activate complement via the alternative pathway. There have been a number of reports showing that the surface cuticle of various nematodes for e.g., *Ascaris* (Leventhal and Soulsby, 1977) *Ancylostoma caninum* (Klaver-Wesseling and Vetter, 1979; Klaver-Wesseling, Vetter and Schoeman, 1982), *N. brasiliensis* (Mackenzie, et al., 1980) and *T. spiralis* (Stankiewicz and Jeska, 1973; Mackenzie et al., 1980) activate complement via the alternative pathway. Other workers have also reported that the cuticle of the trematode *Schistosoma mansoni* may fix complement by the same means (Machado, Gazzinelli, Pellegrino and Dias Da Silva, 1975; Sher, 1976; Tavares, Gazzinelli, Mota-Santos and Dias Da Silva, 1978; Ramalho-pinto, McLaren and Smithers, 1978).
In contrast to the results obtained with NMS, cells adhered to exsheathed L₂ which had been sensitised with IMS in the presence of EDTA although the mean number of cells adhering to each larva was significantly lower than that observed to larvae that had been sensitised with IMS in VBS. In this case the adherence of cells was mediated by specific antibodies, the peritoneal exudate cells adhering to the larvae via their Fc receptors. Antibodies directed against cuticular antigens as measured by cell adhesion have been demonstrated in the sera of animals infected with other nematodes such as *Ascaris suum* (Leventhal and Soulsby, 1976), *A. caninum* (Vetter and Klaiver-Wesserling, 1978), *T. spiralis* (Mackenzie et al., 1978), *N. brasiliensis* (Mackenzie et al., 1980) and *S. mansoni* (Perez and Smithers, 1977; Capron, Dessaint, Capron and Bazin, 1975; Butterworth, David, Franks, Mahmoud, David, Sturrock and Houba, 1977; MacKenzie, Ramalho-Pinto, McLaren and Smithers, 1977; Chung, Asch and Bruce, 1982).

The peritoneal exudate cells associated with the cuticle of the exsheathed L₂ were found to be predominately macrophages which supported the observation reported by Chaicumpa et al., 1977b). However, the observation that peritoneal exudate cells from mice resistant to infection would not adhere to the cuticle of the exsheathed L₂ in the absence of serum is at variance with previous results reported by Chaicumpa et al., (1978) and Prowse et al., (1979b). They showed that macrophages from mice resistant to infection are able to adhere to and damage the parasite in vitro in the absence of serum. Previous investigations by Chaicumpa et al., (1977b) had shown also that damage to the parasite by macrophages required intimate contact between the cells and the cuticle of the parasite. They also provided evidence that the adhesion of cells from immune mice to exsheathed L₂ was due to presence of cytophilic
antibody on the membrane of the cells. A possible reason for
the discrepancy between the present results and those previously
described is that the exsheathed L3 used in these investigations
were different, i.e. the larvae used in the present study were
exsheathed in the mouse stomach under natural conditions. The
previous investigators exsheathed the larvae using NaOCl which may
have caused some damage to the cuticle. It is possible therefore
that the amount of cytophilic antibody associated with the cells
was sufficient to promote their adhesion to the cuticle of such
chemically damaged parasites. Since the present data indicate
that the factors involved in promoting adherence of peritoneal
exudate cells to the cuticle of exsheathed L3 are similar irrespective
of whether the cells were harvested from normal or immune mice, cells
from normal animals were used in further studies.

Further cell adhesion studies demonstrated that all of the
developing post-infective and the adult form of the parasite were
capable of activating complement by the alternative pathway with the
marked exception of larvae collected 96 hours post infection. It
was also noted that the number of cells binding to the larvae
promoted by the complement component, C3, appeared to decrease
quite markedly up to 96 hours post infection when it was almost
nil. It appeared therefore that during the course of larval
development within the host, the ability of the larval cuticle to
activate complement via the alternative pathway changed. A
similar situation such as this has been observed by Mackenzie et al.,
(1980) who showed that the cuticle of the infective larvae and
adult worms of T. spiralis activated complement via the alternative
pathway as indicated by cell binding studies, but the cuticle of
newborn T. spiralis lacked this property. As newborn larvae grew,
however, the newly formed cuticle in the midregion of their body was able to activate complement as measured by cell adherence to this area. The data showed further that peritoneal exudate cells were able to bind to all larval stages and to adult worms of *N. dubius* via their Fc receptors. The binding of cells to 96 hours post infective larvae sensitised with IMS seemed entirely due to binding via Fc receptors. This observation is in agreement with the work of Pritchard and co-workers, (1983) who demonstrated that very few peritoneal exudate cells adhered to post-infective (*L₄*) larvae, which had been recovered 3 days post-infection (using the method of Ey et al., 1981), in the presence of NMS or complement alone, but adherence was greatly enhanced in the presence of heat-inactivated IMS.

During the course of multiple *N. dubius* infections in mice, there was a five-fold increase in the level of IgM and an unusually high IgG₁ level produced (Crandall et al., 1974; Prowse et al., 1978b). The induction of high IgG₁ levels have also been reported for a number of chronic infections caused by metazoan parasites (Mitchell et al., 1977a, b; Sher et al., 1977). Although the immunological importance of this IgG₁ increase is still unclear, the present report showed that IgG₁ played a role in promoting the adherence of normal peritoneal exudate cells to the cuticle of various stages of *N. dubius*. In view of this one might query the suggestion put forward by Mitchell et al., (1977a) that IgG₁ is parasite protective rather than host protective.

The ability of IgM to promote the binding of cells to the larval stages increased dramatically from the pre-infective stages including sheathed and exsheathed *L₃* up to 96 hours post-infective larvae, but promoted little binding to adult worms. The IgG₁
promoted cell binding in a similar pattern to that of IgM, but binding of the cells to 96 hours post-infective larvae was considerably reduced. Adhesion of cells to pre-infective larvae was also enhanced in the presence of IgG\textsubscript{2a} and IgG\textsubscript{2b} but both these immunoglobulin isotypes were not particularly active in promoting cell binding to post-infective larvae. However it should be noted that the amount of IgG\textsubscript{2a} and IgG\textsubscript{2b} required in this reaction were in excess of the physiological levels observed during this infection. At a physiological level only two isotypes, IgM and IgG\textsubscript{1} enhanced cell adherence. Nevertheless it does indicate that the IgG\textsubscript{2a} and IgG\textsubscript{2b} fractions contained specific antibody against cuticular antigens. It seemed likely from the results that the reduced ability of IgM and IgG\textsubscript{1} to promote binding of cells to 96 hours post-infective larvae indicated some change in the antigenic structure of the larvae at this stage.

The expression of stage-specific antigens during the development of nematodes has been described by Mackenzie et al., (1980). They provided evidence for the presence of antigens specific for different stages of \textit{N. brasiliensis} by adsorption of heat-inactivated serum from rats immune to re-infection with infective larvae. The adsorbed serum had a markedly reduced capacity to promote adherence of peritoneal cells to infective larvae while still retaining its ability to promote adherence to adult worms. Likewise adsorption of the serum with adult worms removed the ability of the immune serum to promote cell binding to adult worms but its capacity to promote binding to infective larvae was undiminished. Further they found that an antibody response to infective larvae as measured by cell adhesion was detected from day 5 onwards after infection. The response to adult \textit{N. brasiliensis} was not as great
as that to the infective stage. Antibody could be detected against the adult stage 7 days after infection but had disappeared from the serum after 24 days. In rats infected with *T. spiralis*, Mackenzie and co-workers, (1978) found that the serum contained antibodies directed against the cuticular antigens which were highly specific for each stage in the life cycle. This observation was further supported by Philipp et al., (1981) who demonstrated that serum obtained from rats one week following a primary infection with *T. spiralis* contained antibodies to surface antigens of both infective larvae and intestinal worms but not to surface antigens of newborn larvae as shown by an immunoprecipitation assay. They also showed that there was no cross-reactivity between the antigens of infective larvae and adult worms. Adsorption of immune rat sera with intestinal worms removed antibodies to their surface antigens but not to the surface antigens of infective larvae as measured by an immunoprecipitation assay. Likewise adsorption of sera with infective larvae removed only antibodies to the surface antigens of this stage. These stage specific antibodies were also found to mediate binding of eosinophils to the surface of the larvae. Mackenzie et al., (1980) showed that antibodies induced in rats following infection with either *T. spiralis*, *N. brasiliensis* or *S. mansoni* were highly specific for each species of helminth, as measured by their ability to promote adhesion of eosinophils to the infective stage of all three parasites. The class of antibody which mediated cell adherence and consequent damage to the parasites was not fully defined, but preliminary evidence suggested that IgG2a was involved. It was found also that the cells involved in antibody-mediated damage of newborn larvae (Kazura et al., 1980) and infective larvae (Mackenzie et al.,
1980) of *T. spiralis* were eosinophils. The ability of eosinophils to kill the infective larvae of *T. spiralis* was enhanced in the presence of complement and also when the eosinophil suspension contained neutral red-positive nonadherent macrophages. In *vivo*, these two cell types are often associated, for example, in chronic parasitic granulomas (Warren, Grove and Pelly, 1978). When macrophages and eosinophils were present together *in vitro* the eosinophils adhered to the infective larvae of either *N. brasiliensis* or *T. spiralis* and degranulated. Later the macrophages interacted with the surface of the parasite and phagocytosed eosinophil debris (Mackenzie et al., 1981). Moreover, the macrophages adhered permanently to the surface of these worms, did not flatten and retained their integrity. Under direct interference contrast microscopy the cytoplasmic organelles of macrophages appeared to decrease in size during culture. By transmission electron microscopy both lysosomes and electronlucent structures became increasingly more common in the cytoplasm of cell cultures of macrophages that had been in contact with the parasite for some time.

There are numerous reports in the literature showing that mice may be effectively immunised against *N. dubius* infection by using living *L*₃ but not by using killed *L*₃. However from the present studies show that not only living *L*₃ but also killed exsheathed *L*₃ are capable of evoking antibodies which mediate cell adherence. However despite giving large numbers of killed larvae, the titre of antibodies enhancing adhesion did not reach over the time period studied the levels observed in the serum from mice given living *L*₃ orally. Further antibodies mediating cell adhesion appeared earlier in mice immunised with living *L*₃ than in mice
immunised with killed larvae. Antibodies were demonstrated in the serum 24 days following the oral feeding of living \( L_3 \) at the time when they had received a total of only 400 larvae whereas in the group of mice immunised with killed larvae, antibodies were not detected until 35 days after the first dose at which time the mice had received a total of 10,000 larvae. The data showed also that although mice immunised with killed exsheathed \( L_3 \) had antibodies which mediated cell adherence, these mice were not protected against a subsequent challenge infection with living \( L_3 \) even though they had been given 8 intravenous injections of 2,000 killed larvae. The reason for this is that for good immunity to this infection one requires two effector mechanisms, an 'activated' cellular response as well as the production of humoral antibodies. In general killed vaccines fail to 'activate' the cellular effector arm.

Other investigators have shown that peritoneal exudate cells from normal mice have no effect, either \textit{in vivo} (Chaicumpa \textit{et al.}, 1977b) or \textit{in vitro}, on the infectivity of \( L_3 \) even in the presence of serum from immune animals. In contrast, peritoneal exudate cells from immune mice can damage \( L_3 \) both \textit{in vivo} and \textit{in vitro} (Chaicumpa \textit{et al.}, 1977b; Chaicumpa \textit{et al.}, 1978). The data presented in Chapter 5 support these findings and demonstrate further that peritoneal exudate cells from mice immunised with 2 doses of 200 living \( L_3 \) were able to damage the \( L_3 \) as measured by a loss of infectivity providing the larvae were sensitised with antibody and complement or complement alone. Surprisingly when the larvae were sensitised with antibody alone the larvae were not damaged even though antibody mediated cell adherence. Peritoneal exudate cells from mice immunised with 4 doses of 200 \( L_3 \) were no better at inactivating the larvae than those obtained from mice infected
with 2 immunising doses. The fact that macrophages from normal mice did not harm the larvae even in the presence of both antibody and complement suggests that the macrophages from immunised mice may have been 'activated'. Support for this suggestion was obtained from experiments in which the macrophages had been 'activated' using S. enteritidis 11 RX (Davies and Kotlarski, 1974). The cells from the peritoneal cavity of S. enteritidis 11 RX stimulated mice were able to damage larvae in the presence of antibody and complement or complement alone but not in the presence of antibody alone. Similar results have been obtained by Mackenzie and co-workers, (1980) who found that in the presence of complement alone purified rat macrophages were able to kill the infective larvae of N. brasiliensis following long term culture in vitro. Experiments designed to investigate the killing of protoscolices of Echinococcus multilocularis by peritoneal exudate cells from mice immune to this infection in the absence of serum suggested that the effector cells may be 'activated' macrophages (Rau and Tanner, 1976; Baron and Tanner, 1977) and this activity was enhanced by immune serum. This suggestion was supported by data from experiments which showed that macrophages which had been 'activated' nonspecifically by BCG treatment (Rau and Tanner, 1975; Baron et al., 1977) or by using Taenia crassiceps (Baron et al., 1977) exhibited protoscolicidal activity both in vivo and in vitro. Other in vitro systems have shown that peritoneal exudate cells from normal rats, found to be predominately macrophages were able to mediate in vitro damage to the schistosomulae of S. mansoni in the presence of IgE antibodies (Capron et al., 1975; Capron et al., 1977) or following sensitisation of the cells with heat-inactivated serum from rats immune to S. mansoni (Perez et al., 1977) in the absence of
complement. More recently Auriault and co-workers, (1981) demonstrated that aggregated non-immune rat IgG when added to rat macrophages in vitro induces these cells to become cytotoxic to schistosomula providing guinea pig complement was added to the culture medium.

The expression of resistance to an *N. dubius* infection may involve cells other than activated macrophages since Penttila (personal communication) has shown that both eosinophils and neutrophils are able to damage exsheathed *L₃* in vitro in the presence of antibody and/or complement. It is interesting to note that neutrophils from normal mice are inactive under similar conditions.

Previous investigators have with one exception reported that immunity to *N. dubius* infections cannot be transferred passively by immune serum (Panter, 1969; Cypess, 1970; Chaicumpa et al., 1976; Chaicumpa et al., 1978; Prowse et al., 1978b). However in the present study it was found that incubation of exsheathed *L₃* with serum freshly collected from mice infected with 4 doses of 200 *L₃* resulted in larval damage as measured by a reduction in their infectivity. Based on these results it seemed possible that following several infection a larvicidal factor(s) was produced by the mouse and this may play a role in vivo in the development of host resistance.

Experimental data revealed that larvae which had been exposed to IMS(4) for only one hour, washed and then incubated further in supplemented medium A for up to 24 hours were impaired in their infectivity to the same extent as if they had been in contact with IMS(4) for 24 hours. However it was clear that damage to the parasite took some time to develop since larvae which had been in contact with IMS(4) for up to six hours were not impaired in their infectivity. It is also interesting to note that only a percentage
of the larvae appeared to be sensitive to the larvicidal activity of the IMS(4). Further addition of either IMS(4) or complement to larvae during the incubation period did not increase their loss of infectivity compared with those that had been incubated with a finite amount. The data in Chapter 6 showed that the larvicidal activity of IMS(4) was heat labile which indicated that complement could be playing a role. However the activity could not be restored by the addition of fresh NMS as a source of complement. Later it was shown that the larvicidal activity of IMS(4) destroyed by methylamine could be restored by the addition of fresh NMS as a source of complement. It was possible that heat treatment not only inactivated complement but if specific immunoglobulins were involved in the larvicidal activity of IMS(4) these may have been denatured. Methylamine however attacks a unique, labile thioester bond in C2, C4 and 

\[ 2 \text{-macroglobulin} \] by nucleophilic substitution and does not affect immunoglobulins. The data showed also that this larvicidal activity was found only in long term \textit{N. dubius} infected mice since serum from mice infected with \textit{M. corti} an unrelated helminth parasite did not reduce the infectivity of exsheathed \textit{L}_3 of \textit{N. dubius}. The fact that IMS(4) lost its activity after storage on ice for several days and following freezing and thawing again implicated complement as one of the factors since mouse complement is particularly labile to such treatments. Further confirmation of the role of complement in this reaction was obtained when it was shown that IMS(4) that had been passed down a rabbit anti-mouse C3 affinity column had completely lost its larvicidal activity. Such activity could be restored by the addition of NMS to the chromatographed fraction. Initial experiments indicated that immunoglobulins may not be involved in the larvicidal activity since passage of
the IMS(4) down an anti-mouse F(ab')2 column did not diminish its activity at the dilution tested. However an ELISA assay showed that only 90% of the immunoglobulins had been removed and so the activity could have been still due to the remaining 10%. Further studies using purified IgG and IgM fractions from IMS(4) showed that the IgM fraction in the presence of complement was capable of damaging the larvae whereas the IgG fraction was not. This finding would help to explain the observation that IMS(4) heated at 56°C for 30 minutes had lost its larvicidal activity and this could not be restored by the addition of IMS as a complement source, since mouse IgM is relatively heat labile.

In the present study experiments in vivo demonstrated that when serum from mice immunised with 4 doses of 200 L3 was given intravenously to naive mice there was a significant transfer of protection when these mice were subsequently challenged within a short space of time. Transfer of serum from mice previously infected with 2 immunising doses or from normal mice had no effect. It should be noted that in this present study the larvae were given intravenously after the administration of IMS(4) and not by the normal oral route used in previous studies mentioned before where various investigators had failed to demonstrate immunity by such means.

However since N. dubius infections in mice were thought to be a useful model for human hookworm infections it seemed not unreasonable to challenge them by this route. The means by which mice were protected following the passive transfer of IMS(4) remains to be elucidated but it is clear it does not involve 'activation' of phagocytic cells.

The in vitro observations concerning the factors involved in promoting cell adherence show quite clearly that these are specific
antibody and/or complement. However although cell adhesion is promoted by specific antibody alone this does not lead to damage of the larvae as measured by a loss of infectivity. In contrast in the presence of complement alone, larval damage occurs if the macrophages are obtained from immune mice, and as other investigators have reported are in an 'activated' state (Chaicumpa et al., 1978). Similar observations have been made using neutrophils. Neutrophils from mice resistant to re-infection may bind to the larvae via antibody and/or complement (Penttila, Ey and Jenkin, 1983) and like macrophages are unable to damage the larvae in the presence of antibody alone but will do so if complement alone is present (Penttila personal communication). As with the case of macrophages, neutrophils from normal mice although adhering to larvae under the above conditions do not express larvicidal activity. Thus it might appear that specific antibody in vivo is not an important pre-requisite for the development of resistance of mice to this infection but immunity depends on the induction of a change in the ability of phagocytic cells to become larvicidal. However this appears not to be the case. Infection of mice with S. enteritidis 11 RX while it leads to the production of altered macrophages and altered neutrophils (Chaicumpa et al., 1978; Penttila personal communication) which in vitro are larvicidal given the appropriate conditions described above, does not give rise to an increased resistance to N. dubius. Nevertheless if to such S. enteritidis 11 RX infected mice, serum from mice immune to re-infection with N. dubius is transferred passively and the mice are challenged orally with infective L3 then a high degree of protection is achieved. It should be noted that the IMS did not endow any protection on uninfected mice (Jenkin personal communication). It is clear therefore that there is a discrepancy between the in vitro and in vivo findings which present a paradox.
A possible explanation for these apparent contradictory results may be as follows.

Phagocytic cells capable of damaging larvae need to bind to the parasite via their C3 receptors in order to trigger off their cytocidal mechanisms; binding through their Fc receptors is not sufficient to do this. Thus in vitro in the absence of antibody where complement is not limiting the phagocytic cells can adhere and express their larvicidal potential. The in vivo situation is somewhat different. The larvae in immune mice are killed while developing in the muscularis mucosa. There is a marked inflammatory reaction around the developing larvae and it is possible because of the degree of tissue damage, complement may be inactivated and consequently in limiting amounts on the surface of the larvae. In mice treated with S. enteritidis Rx therefore while the phagocytes are potentially larvicidal, they are unable to express this potential due to the limiting amount of complement. If one however transfers passively IMS binding of the cells to the larvae could take place via their Fc receptors and it is plausible that the small amount of C3 associated with the larvae may be sufficient to trigger the cells. Obviously this may not be the only explanation but it does open the way to further experimentation.
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