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THE MECHANISMS INVOLVED IN IMMUNITY TO *Nematospiroides dubius* (BAYLIS, 1926) INFECTION IN MICE

by

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ABSTRACT

In a recent review Ogilvie and Jones (1973) have highlighted the fact that progress on acquired immunity to helminths has been remarkably slow and in most instances we are still not sure of the relative importance of antibody in immunity to these infections or of the role played by cells such as macrophages and lymphocytes. One of the reasons for the slow progress has been due in part to the complicated life cycles of many of these parasites which makes them difficult to study in the laboratory.

We have chosen to study the mechanism of acquired immunity to a nematode parasite (*Nematospiroides dubius*) which has a relatively simple life cycle and which is a natural parasite of the mouse.

Initial studies showed that the mouse may acquire active immunity against the parasite following the intravenous injection of living infective third stage larvae. Killed larvae are ineffective. The presence of specific antibody may be measured in the circulation of the host after immunisation. All attempts to transfer immunity passively with serum failed although it was possible to demonstrate the transfer of immunity passively from immune mothers to their offspring.

The data obtained from infection studies in immune mice indicate that the acquired immunity is directed against the infective third stage

larvae at the time it penetrates the wall of the intestine prior to encystment and not against the adult stage which lives free in the lumen of the small intestine.

Experiments both *in vivo* and *in vitro* indicate that immunity to this infection requires the 'activation' of certain cells which are able then to kill the larvae. The *in vitro* studies show that the cell is an adherent cell and is possibly therefore a macrophage. Recognition of the parasite seems to be mediated by trypsin labile receptors on the surface of the cells. Trypsin treated cells from immune animals are unable to inactivate the parasite though this ability is restored in the presence of specific antibody.

The results presented in the thesis favours the idea that acquired immunity requires the co-operation between an 'activated' cell and specific antibody.

Declaration

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 when reference is made in the text.

(V. Chaicumpa)

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CHAPTER 1
INTRODUCTION

1. GENERAL INTRODUCTION

Diseases caused by metazoan parasites have played an important role in the development of human communities. Although both in man and animals infection with many of these parasites is seldom fatal, it does lead to a chronic debilitating illness, which poses many public health problems and economic problems associated with livestock management. Infection with helminths is a good example of such diseases and will be the subject of this review, with particular reference to nematode infections.

Knowledge regarding many aspects of diseases caused by helminths has increased in recent years. For example, there is a better understanding of the host-parasite relationship and the physiology of these parasites. Drugs for effective treatment and protective vaccines have been developed such as vaccines against lung worm in cattle and sheep (Jarrett, Jennings, McIntyre, Mulligan and Urquhart, 1960; Jovanovic, Sokolic, Movsesijan and Cuperlovic, 1965), against hook-worm in dogs (Miller, 1965) and tracheal nematodes in chickens (Varga, 1968). There are however still many problems associated with diseases caused by nematodes about which little is known or about which there is a great deal of controversy, in particular the mechanism involved in immunity to these parasites, and the antigens which may play an important role in inducing host immunity.

2. ANTIGENS OF THE NEMATODES

In spite of extensive studies little is known about the antigens of nematodes. One of the major difficulties has been due to the complex nature of the parasite e.g. the complexity of their somatic structures, their many stages of development, the changes in their biochemical and physiological products, all these factors make the study of antigens which may be involved in inducing host immunity very complicated (Sinclair, 1970). However, despite these difficulties some "protective" antigens have been defined (Chandler, 1935; Thorson, 1953; Weinstein, 1959; Mills and Kent, 1965 and Ogilvie and Jones, 1971). Unfortunately in many instances these "protective" antigens represent only minor structural components and the yield is thus correspondingly small (Thorson, 1970).

The antigen of nematodes may be classified into two main groups:

i.e. the somatic (structural) antigens
the exoantigens or physiological antigens.

2.1 Somatic antigens of nematodes

The somatic antigens of nematodes have recently been reviewed e.g. by Thorson (1970), Sinclair (1970), Fife (1971) and Ogilvie and Jones (1971). These antigens may be prepared from the parasites at different stages of development using different procedures for extraction. Melcher (1943) obtained albumin-like and globulin-like antigens from adult trichinellae (Trichinella spiralis) by grinding up the whole worms, removing the lipids with ether and recovering the two types of antigens

on the basis of their solubility in acid. Polysaccharide antigens have been prepared also from nematode parasites, such as ascaris, by repeated precipitation of borate buffer extracts of the whole adult worms with cold ethanol (Campbell, 1936; Oliver-Gonzalez, 1946). Oliver-Gonazalez (1943) and Kagan (1957) prepared protein antigens from Ascaris lumbricoides by dissecting off the cuticle and muscle of these animals and extracting these tissues with saline.

It is obvious from the above that these antigenic preparations were crude and in many cases ill-defined, for example as globulin-like or albumin-like proteins.

Recently Kent (1960) using the technique of gel-filtration isolated at least 6 different proteins from a distilled water extract of fresh female adult Ascaris lumbricoides (var. suum). Prior to chromatography the lipids had been removed using absolute ethanol and ether at -70°C.

Hogarth-Scott (1967) employing Sephadex G25 G50 G75 isolated a variety of antigens differing in molecular weight from the body fluids of ascaris and from saline extracts of adult Nippostrongylus, Toxocara and Toxocaris which were able to elicit passive cutaneous anaphylaxis reactions in man, rabbits, dogs and cats infected with these parasites. Unfortunately in most cases these involved protein or pholysaccharide antigens have not been used to immunise the animal against the specific infection but have been employed as a diagnostic tool in skin tests which measure the extent of exposure of a community of man or other

animals to a particular nematode infection.

2.2 Exoantigens or Physiological antigens of nematodes

Studies have shown that materials are produced and excreted from helminths during development. These are known as exogenous metabolic products or physiologic products. These materials react strongly with serum taken from their respective infected hosts (Taliaferro and Sarles, 1939; Otto, 1940; Mauss, 1941; Sadun, 1949; Oliver-Gonzalez, 1940). The ability of these exoantigens to stimulate effective immunity in the host has been studied also (Thorson, 1953; Soulsby, 1957; Mills and Kent, 1965).

Thorson (1953) collected excretions and secretions (ES antigens) from larvae of *Nippostrongylus brasiliensis*. Such preparations when used to immunise rats protected these animals from subsequent infection with this parasite, as shown by a 50-60% reduction in the number of adult worms in the intestine when compared to unimmunised controls. The antigens contained, in the excretions and secretions, a lipolytic activity. The other nematode from which ES antigens have been isolated is *Ascaris suum.* Rhode, Nayak, Kelley and Marsh (1965) isolated malic dehydrogenase from adult worms by extracting them with a Tris (hydroxymethyl aminomethane) - HCl buffer, at pH 8.5 or with 0.087M acetic acid titrated to pH 6.0 with 3N NH_4OH. Fractionation of the extract was performed on a DEAE-cellulose column. They found that the fraction containing malic dehydrogenase activity antigen conferred an appreciable protection (about 50% reduction

of larvae recovered in the lungs compared to the controls) on guinea pigs against this infection, whereas the other fractions devoid of this enzyme activity were inactive.

The other sources of exoantigens are hatching fluid and moulting or exsheathing fluids. Stewart (1953); Soulsby, Sommerville and Stewart (1959), and Soulsby and Stewart (1960) have found that the fluids released during the third moult of Haemonchus contortus were the essential stimulus for the termination of the infection in sheep. Injecting this fluid into the abomasum of sheep carrying the adult worm led to an anaphylactic type reaction in this organ which resulted in the expulsion of the adult worm. The agar-diffusion precipitin reactions were pronounced when these antigens were used with sera from infected sheep during the self-cure phenomenon.

Sommerville (1957) and Rogers and Sommerville (1960) studied the nature of the exsheathing fluid of Haemonchus contortus and found that this material contained low molecular weight proteins. The hatching fluid of Ascaris lumbricoides has been studied by Rogers (1963) and found to contain substances with chitinase and esterase activities. However, these workers did not study the protective effect of immunising animals with these materials on subsequent infection.

There are many antigens of nematodes which have the capacity to stimulate the host to produce antibodies. However, antibody production does not alway correlate with the resistance of the host to infection. Many workers have tried to isolate or identify so-called "critical antigens"

e.g. specific antigens, which may be used as vaccines conferring protection on the host to infection, in contrast to other antigens from the same parasite which do not, even though they may be immunogenic.

The observation of Chandler (1935) that antigens which stimulate immune mechanisms of the host against nematode parasites are substances released by living worms has been investigated further. Sarles (1938) was the first to demonstrate the formation of precipitates at the oral pore, excretory pores and anal opening of N. brasiliensis during incubation with antiserum from infected rats. Similar results with the same parasite were reported using histological techniques (Sarles and Taliaferro, 1936).

Thorson (1953) demonstrated that a vaccine prepared from the culture fluid (normal rat serum and normal saline) in which the infective larvae of N. brasiliensis had been maintained conferred a significant degree of protection on rats. The adult worm recovery from the intestine of the immune rats was 50-60% lower than in the controls. The lypolytic activity in that culture fluid was inhibited by serum from vaccinated rats. Thorson (1956a, b) working with Ancylostoma caninum demonstrated that saline extracts from oesophageal glands, which possessed proteolytic activity, produced a low degree of protection ($P < .05$ to $.01$) in puppies against subsequent infection after vaccination with this extract, as judged by the number of adult worms recovered and their size.

Guinea pigs infected with Ascaris suum were studied by Rhodes

et al. (1965). These workers showed that the vaccination of guinea pigs with malic dehydrongenase isolated from adult *Ascaris suum* as described in Section 2.2 appeared to give significant protection against migrating larvae. There was a 50% reduction in the number of larval stages in the lungs when compared to unvaccinated controls. Other investigations have demonstrated also that the metabolic products from second, third or fourth stage larvae of *Ascaris suum* will protect animals against this infection as judged by the reduction of larval numbers in the lungs of immunised animals, Soulsby (1957, 1963); Arean and Crandall (1961, 1962), Crandall and Arean (1965) and Guerrero and Silverman (1969, 1971). In contrast the somatic antigens isolated from the larval stage of this nematode have been found by Crandall and Arean (1965) to be ineffective in inducing immunity. Recently Guerrero and Silverman (1971) have shown that metabolic and somatic products of second and third stage larvae of *A. suum* from a 12 day old *in vitro* culture were capable of inducing resistance to this infection in mice.

In a similar study with *T. spiralis* infection in mice Mills and Kent (1965) demonstrated that a rabbit antiserum against ES antigens from larvae, when preincubated *in vitro* with the larvae for 48 hours, reduced their infectivity by more than 50%. However, the nature of the antigens was not known. Despommier and Muller (1970) have shown that mice can be immunised against *T. spiralis* with granules obtained from the multi-cellular stichosome. The pure protein from this organ gave an 80-98% reduction of muscle larvae when it was used to immunise mice.

The evidence that protective antigens are either released from or present in adult worms of N. brasiliensis (Denham, 1968, 1969) has been discussed by Ogilvie and Jones (1971, 1973). Ogilvie and co-workers suggested that antibodies against enzymes other than the lipase (Thorson, 1954) may be important in the immunity to this nematode. Their investigations suggested that antibody directed against acetyl-cholinesterase may play an important role in host immunity.

It is clear from these varied studies that the term protective antigen is almost meaningless and a great deal more work needs to be done on the protective efficacy of well-defined antigens obtained from these parasites.

3. MECHANISMS OF IMMUNITY TO NEMATODES

Our knowledge of the mechanisms of immunity to nematode parasites has been increasing over the last 40 years. Numerous workers have investigated such infections as trichinosis, ancylostomiasis, haemonchusiasis, trichostrongyliasis, ascariasis and in particular Nippostrongylus brasiliensis infections in rats and Nematospiroides dubius infections in mice. These latter two infections have been useful laboratory models not only in studying the life-cycle of these parasites but also in studying the humoral and cellular factors involved in immunity. It is pertinent therefore to review what is known about the mechanisms thought to be involved in the development of host resistance to infection by these parasites.

Nippostrongylus brasiliensis is a representative of the skin penetrating type nematodes which have a short life span. The natural host is the rat which can be infected by allowing either the third stage larvae to penetrate the skin, or by injecting infective larvae subcutaneously. The larvae develop and migrate through the lungs and finally become adult in the lumen of the small intestine about 5-6 days after infection. The adult worm is expelled by the host about 14-15 days later.

The elimination of adult worms from the rat has been investigated by Jarrett, Jarrett and Urquhart (1968) and can be divided into four phases followed the initial infection:

a) The "loss phase one" due to the failure of infective larvae to continue development after penetrating the skin, b) the "plateau phase", during which surviving larvae develop to maturity in the small intestine, c) the "loss phase two", when the adult worms are rapidly expelled from the small intestine, and finally d) the "threshold phase" during which a residual number of adult worms survive.

It has been suggested that the "loss phase one", in which 40-60% of the larvae may die, is due to some specific anti-helminth factor(s) unrelated to immunoglobulin.

The "loss phase two", which is due to acquired immunity is of more immediate importance here. A similar pattern of termination of infection is found with other nematodes e.g. Dictyocaulus viviparus in guinea-pigs (an abnormal host, Wilson 1966); Ostertagia circumcincta in sheep and

cattle (Amour, Jarrett and Jennings, 1966); and in sheep infected with *Haemonchus contortus* (Stoll, 1929). In some of these infections the elimination of adult worms does not occur unless the host becomes reinfected with the same parasite. Stoll (1929) termed this the "self-cure" phenomen and demonstrated it in sheep infected with *H. contortus*. The "self-cure" is characterized by a rapid loss of adult worms following a superimposed infection with infective larvae. Animals, which had expelled their worm burdens, were found to be resistant to further infections. Studies by Stewart (1950a, 1950b) demonstrated that this phenomenon could be produced in the laboratory. In 1953 Stewart's results led him to the conclusion that the expulsion of adult worms of *H. contortus* in sheep was due to a local hypersensitivity reaction of the immediate type which occurred in the abomasal mucosa following the invasion of the host with the second dose of infective larvae, the host having been sensitized by the primary infection.

The expulsion of adult worms in infections with *N. brasiliensis* in rats is also regarded as a "self-cure" phenomon although it does not depend upon the animals being reinfected with infective larvae as in the case of *H. contortus* (Mulligan, Urquhart, Jennings and Neilson, 1965; Barth, Jarrett and Urquhart, 1966). The immune mechanisms of the host may act upon larval stages as well as on the adult worms. The consequence of these reactions may be measured in various ways such as the retarded development of the parasites e.g. *Nematodirus spathigen* and *H. contortus*

in sheep (Donald, Dineen, Turner and Wagland, 1964; Dineen, Donald, Wagland and Offner, 1965); the decreased egg production by adult female worms e.g. N. brasiliensis in rats (Chandler, 1932), abnormal anatomical features e.g. lack of vulval flap in female O. ostertagi in calves (Michel, 1967); physiological changes such as the decreased acetylcholinesterase content in N. brasiliensis in immunised rats (Ogilvie and Jones, 1971).

It is possible that host mechanisms other than the classical immune response may inhibit the development of nematodes and other helminths. Indeed this possibility has led to a great deal of controversy among workers considering factors involved in host resistance.

A good example to illustrate this concerns the trichostrongyle parasites of sheep. The development of the larvae of several species, which are picked up by grazing animals in the autumn, is often arrested when the fourth stage is reached in the mucosa of the abomasum or small intestine. The parasites remain in a state of arrested development and become adult in the spring. It has been suggested that this arrested development is immunological or due either to changes in the nutritional status of the host or to changes in the level of certain hormones which are important in stimulating larval development (Dineen, Donald, Wagland and Offner, 1965; Donald, Dineen, Turner and Wagland, 1964; Nelson, Blackie and Mukundi, 1966; Poechel and Todd, 1969; Blitz and Gibbs, 1972, 1972a; Brunsdon, 1973 and McKenna, 1973).

3.1 The role of antibody in host immunity to nematode infections

It is now well established that specific antibody plays a major role in protecting animals from bacterial infections, whether these bacteria be extracellular or intracellular parasites. Bacteria that are intracellular parasites pose special problems because the destruction of these bacteria, once they have been engulfed by phagocytic cells, may depend on an altered physiological state of these cells. The precise mechanism by means of which the phagocytic cells, namely macrophages, become "activated" is not clear though evidence points to the involvement of specifically sensitized small lymphocytes. This matter will be considered in more detail later. However, what is clear is that the increased bactericidal potential of these cells may only be expressed if the bacteria have been in contact with specific antibody. In the absence of specific antibody bacteria are not recognised by the phagocytic cells. (Mackaness, 1962, 1964; Jenkin and Rowley, 1963; Rowley, Turner and Jenkin, 1964; Mackaness, Blanden and Collins, 1966; Ralston and Elberg, 1969).

It is difficult with such large parasites as the helminths, to visualise host immunity involving interaction of the parasite with specific antibody followed by ingestion by phagocytic cells. However, evidence in the literature indicates that specific antibodies are important in determining resistance to these infections, although the mechanisms involved are the subject of a great deal of controversy.

Chandler (1938) and Sarles (1939) showed that rats could be protected against infection with *Nippostrongylus muris* if they had been injected prior to challenge with serum obtained from infected rats. Similar results have been obtained with other nematode parasites such as *Strongyloides ratti* and *Trichinella spiralis* (Lawler, 1940; Culbertson, 1942). In general the protection obtained by such means has been poor and in many cases very large amounts of serum were required to produce an effect. Miller (1967) showed, for example, that he could protect puppies against *Ancylostoma caninum* by passive transfer of serum from immune animals but in order to demonstrate such immunity the total amount of serum given in a series of doses was twice the blood volume of the puppy.

There are a number of reasons why negative results obtained from the transfer of immunity by serum are difficult to interpret when one is considering the protective role of antibody. In many cases no consideration has been given to the half-life of the transferred immunoglobulins in relationship to the duration of the infection or the dilution effect involved in passive transfer. For this and other reasons many recent investigators have studied this problem using immunoglobulin fractions obtained by various chromatographic techniques (Ogilvie, 1970).

3.2 Classes of immunoglobulin and their roles in immunity to nematode infections

In general, during an infection, antibodies to antigens of

nematodes may be found in all the major classes of immunoglobulins.

Wilson (1966) showed that the IgG_1 fraction of serum from guinea-pigs infected with the cattle lung worm *Dictyocualus viviparus* would protect normal animals from infection with this parasite as shown by a 50% reduction in the number of worms recovered from the passively immunised animals. Jones, Edwards and Ogilvie (1970) demonstrated the protective effect of serum from rats that had been repeatedly infected with *N. brasiliensis* infective larvae. The serum was fractioned by gel filtration on Sephaded G-200. They found that the IgG_1 fraction of the serum conferred protection when passively administered to normal animals whereas the IgM and IgA fractions were ineffective. Antibodies of the IgG_2 class were found also to be ineffective. All these fractions were free from reaginic activity. In contrast Catty (1969) who studied the immune response of guinea-pigs to *Trichinella spiralis* has suggested that the protective immunoglobulins were in that fraction of the serum which possessed reaginic activity.

Perhaps the most controversial area which exists in studies on immunity to nematode infections is the role that reaginic antibody plays in host resistance to infection. It is well known that infections with helminths may initiate hypersensitivity reactions in both man and animals. Indeed the immediate type hypersensitivity reaction has been used to diagnose such diseases as ancylostomiastis, trichinosis, and ascariasis (Bruner, 1928; Coventry and Taliafero, 1928; Oliver-Gonzalez,

1946; Sprent and Chen, 1949; Sadun, Buck and Wattson, 1959; Woodruff, Thacker and Shah, 1964; Johansson, Mellbin, and Vahlquist, 1968 and Hogarth-Scott, Johansson and Bennich, 1969). The antibodies involved in this reaction are analogous to human IgE (Ishizaka, Ishizaka and Hornbrook, 1966a, 1966b; Ishizaka, 1969).

Rats infected with N. brasiliensis produce reagnic antibody (Ogilvie, 1964) and provide a good model for the study of the role of reagins in host immunity to nematode infections. The reaginic antibody which has been described as heat- and mercaptan-labile could be detected in the serum of rats three weeks after the initial infection (Jones and Ogilvie 1967a;Broch and Wilson, 1968). The titre remained high for 3 to 6 days and then fell to a low level. Urquhart and co-workers have attempted to show that reaginic antibody reacting with antigens from the worm results in an immediate hypersensitivity reaction which leads to worm expulsion (Urquhart, Mulligan, Eadie and Jennings, 1965; Mulligan, Urquhart, Jennings and Neilson, 1965; and Barth, Jarrett and Urquhart, 1966).

The reasons why an immediate hypersensitivity reaction should result in the host ridding itself of its worm burden is obscure. The above workers have suggested that the hypersensitivity reaction gives rise to lesions on the epithelium of the gut which, due to an altered permeability, allows other specific antibodies to leak through into the lumen and react with the worms. Ogilvie and other workers have shown

that specific antibody against N. brasiliensis will damage adult worms as indicated by the pathological changes in their gut (Ogilvie and Hockley, 1968; Lee, 1969; Edwards, Burt and Ogilvie, 1971).

Support for the importance of an immediate type hypersensitivity reaction in determining host immunity to these infections has come from a number of studies involving the use of antihistaminic drugs during the course of the infection.

Chlorpheniramine, and anti-5-hydroxytryptamine agent (1-benzyl-2-methylmetoxy tryptamine or BAS), prolonged the intestinal phase of Trichinella spiralis infection in mice (campbell, Hartman and Cuckler, 1963). Rothwell, Dineen and Love (1971), and Rothwell, Prichard and Love (1974) demonstrated that antihistamine and antiserotonin drugs inhibited the expulsion of Trichostrongylus colubriformis. Recently, Kelly and Dineen (1972) suggested that promazine hydrochloride may inhibit worm expulsion in rats infected with N. brasiliensis, possibly by an effect on lymphocyte function rather than on cells which release histamine.

Murray (1972) has proposed that specific IgE antibody on mast cells leads to degranulation of these cells following the reaction of this class of immunoglobulin with worm antigens. The released pharmacologically-active mediators cause disruption of the plasma cells in the surrounding area which allows a high concentration of specific IgG antibody to react with the worms.

However in contrast to these studies other investigators who have suggested that reaginic antibody and immediate type hypersensitivity

reactions are relatively unimportant in determining the resistance of the rat to infection with *N. brasiliensis*.

Jones and Ogilvie (1971) have proposed that worm expulsion occurs in at least two stages. In the first stage specific antibody damages the worms possibly by combining with enzymes essential for the nutrition of these parasites. The second stage which involves the expulsion of the worm burden seems to be sensitive to irradiation, a factor which will be enlarged upon in the following section.

3.3 Cell mediated immunity in nematode infections

Cell mediated immunity in nematode infections has not received much attention in the past. Cellular responses to these parasitic infections have been dismissed as secondary, non-specific phenomena associated with the primary reaction of antibodies with the parasites. More recently the possible active participation of cells in determining resistance to infection by helminths has received attention.

Studies by Wagland and Dineen (1965) and Dineen and Wagland (1966) indicated that certain cells may play an active role in worm expulsion. Using guinea-pig infected with *Trichostrongylus colubriformis* they demonstrated that immunity can be transferred with lymphocytes from the mesenteric lymph node of the immune host but not with immune serum. Dineen, Ronai and Wagland (1968) also found that the transferred lymphocytes which accumulated at the site of infection in the small intestine, and which came into intimate contact with the parasite in

the epithelium, promptly underwent "allergic death" or lysis. Further studies by Dineen and Adams (1971) indicated that long-lived small lymphocytes were the effector cells in determining immunity. They demonstrated that neonatally-thymectomised guinea pigs that had undergone long term lymph drainage were unable to eliminate this parasite. One could argue that such treatment would effect markedly the ability of the animal to produce specific antibody. However failure to transfer immunity with a specific antiserum would suggest rather the direct involvement of sensitised lymphocytes in determining resistance to this infection, although it does not rule out a cooperative mechanism involving specific antibody and these cells. Other similar studies with N. brasiliensis and Ostertagia circumcincta have again emphasised the role of sensitised lymphocytes (T cells) in determining immunity to these infections (Ogilvie and Jones, 1967; Anderson, Curtain, Johnson and Simons, 1972).

As mentioned previously Jones and Ogilvie (1971) showed that adult worms of N. brasiliensis affected by antibodies are expelled from rats by a radiosensitive factor. Keller and Keist (1972) confirmed these observations and found that this factor could be restored by giving irradiated animals mesenteric lymph node cells from normal animals. However in order to get an accelerated rejection the worms in the reconstituted rats had had to be in contact with specific antibody. Extending these studies Dineen, Ogilvie and Kelly 1973, found that irradiated rats that had received mesenteric lymph node cells from immune animals but which were challenged with normal worms transplanted into

the intestine, expelled the parasites prematurally but not completely. In contrast, in irradiated rats given similar treatment but challenged with worms that had been in contact with antibody, the expulsion was rapid and complete. Dineen *et al.* (1973) did not think that the difference in time of expulsion between the former group of rats and unirradiated individuals showed that cells from immune animals affected the worms *per se*. They suggested that the difference was a reflection of the rate of antibody production and cell sensitisation. In the irradiated animals given cells from immune individuals one arm of the effector mechanism was already developed and merely required the presence of specific antibody to become fully functional.

In terms of the mechanisms of immunity to helminth infections these studies are of great importance, particularly for the development of efficient vaccines. It is clear if an effective killed vaccine is to be developed it must stimulate both the cellular and humoral arms of the immune response.

Because this thesis is concerned with the mechanism of immunity to *Nematospiroides dubius* it might be pertinent now to describe the life cycle of this parasite and what is known about the development of resistance to this infection.

3.4 Life cycle of *Nematospiroides dubius*

Nematospiroides dubius (Nematoda, Strongyloidea), a parasite of mice, was first described by Baylis (1926). The life-cycle of this

nematode, which is direct, has been investigated by several workers e.g. Spurlock (1943); Ehrenford (1954); Baker (1954, 1955) and Fahmy (1956). The first stage larvae hatch from the egg and moult to form the second stage larvae. The second moult occurs and the larvae reach the third stage about 6 to 7 days after hatching. The third stage larva is enclosed in the partly cast cuticle or "sheath" of the second stage larva. The third stage larva is the "infective" form and may infect several species of rodents, though the mouse is the most susceptible (Cross, 1960; 1964). From the time of hatching to the third stage the parasite develops as a free-living form. The parasitic stage starts on ingestion of the third stage larva by the host. The exsheathing or the completion of the second moult takes place in the stomach and the larvae become attached to the gastric mucosa within a few hours of ingestion (Liu, 1965a; Panter, 1967). They remain in the stomach for approximately 36 hours and then pass into the small intestine. In the small intestine the larvae invade submucosa and muscularis externa where encystment occurs. This takes place approximately 96 hours after infection. Within the cyst the larvae again moult to produce a fourth stage larva which develops into the young adult. After a period of from 2-4 days the young adult worm vacates the cyst and migrates to the small intestinal lumen. Here mating occurs and numbers of eggs appear in the faeces of the mice. The whole life-cycle illustrated in Figure 1.1, takes about 15 days. The prominent exposure of this nematode to host tissues occurs during the migration of

the third stage larva through the intestinal mucosa, and when the young adult worm migrates from the cyst to the intestinal lumen.

The pathogenic changes that take place in the small intestine during infection with this nematode have been investigated by Baker (1954, 1955) and Liu (1965a, b). During penetration the larvae caused petechial and chymotic haemorrhages extending from the pylorus to the duodenal part of the small intestine. The wall of the small intestine appeared thinner, the lumen was dilated and splenomegaly had occurred. These changes were increased markedly on day 4 (Baker, 1954). Diarrhoea occurred at this time and there was an increased volume of fluid in the peritoneal cavity on day 5 and day 6. Microscopic examination of sections of the intestine showed the presence of a marked cellular infiltration during the emergence of the young adult worm from the cyst. The types of cells involved were neutrophils, macrophages, plasma cells, lymphocytes and a few eosinophils. Lymphadenitis and hyperplasia of the reticuloendothelial tissue in the mesenteric lymph glands, and non-specific hepatitis was also found. In subsequent re-infections these changes were more severe than those found in the primary infection.

3.5 Immunity in mice to infection with *Nematospiroides dubius*

The development of resistance to *N. dubius* infection after infection with third stage larvae has been studied by various workers. Van Zandt (1961) found that mice that had been given three doses of

50 third stage larvae orally, displayed some resistance to further re-infection as measured by the number of adult worms in the intestine of these animals compared to the controls after challenge with a standard dose of larvae. Other workers have shown using the above criteria of immunity that the degree of resistance is dependent on the number of infective larvae used in the immunising dose or doses (Panter 1967, 1969a; Bartlett and Ball, 1972; Hosier and Feller, 1973). Effective immunity may also be conferred on mice by injecting them subcutaneously with 4000 exsheathed larvae (Lueker, Rubin and Anderson, 1968; Rubin, Lueker, Flom and Anderson, 1971). Panter (1969b) has suggested that the resistance is dependent on an immediate type hypersensitivity reaction which occurs after the administration of the challenge dose of larvae to mice previously immunised with third stage larvae of the same species. The anaphylactic reaction taking place in the gut prevents the establishment of the larvae in the wall of the intestine. Some support for this hypothesis stems from Panter's observations, that mice made hypersensitive to horse serum were more resistant to infection with third stage larvae than normal mice, providing that the sensitised animals were given horse serum, which provided an anaphylactic reaction of varying severity, at the time of challenge. In contrast Cypess (1970) has suggested that a delayed type hypersensitivity reaction involving sensitised lymphocytes is the effector mechanism. Experiments performed by Ninnemann (1971) using millipore chambers planted in the peritoneal cavities of normal and

immune mice suggest that macrophages, in collaboration with specific antibody, constitute the important immune mechanism conferring resistance to this infection.

It is clear from the literature that prior exposure of mice to the third stage larvae of *N. dubius* protects them from subsequent re-infection. However the mechanism determining resistance is far from clear. The purpose of the present work was an attempt to define this mechanism in more detail.

FIGURE 1.1

The life cycle of *Nematospiroides dubius* showing the non-parasitic or free living stages and parasitic stages.

L1, L2 and L3 = First, second and third stage larvae.

STAGES IN THE LIFE-CYCLE OF *Nematospiroides dubius*

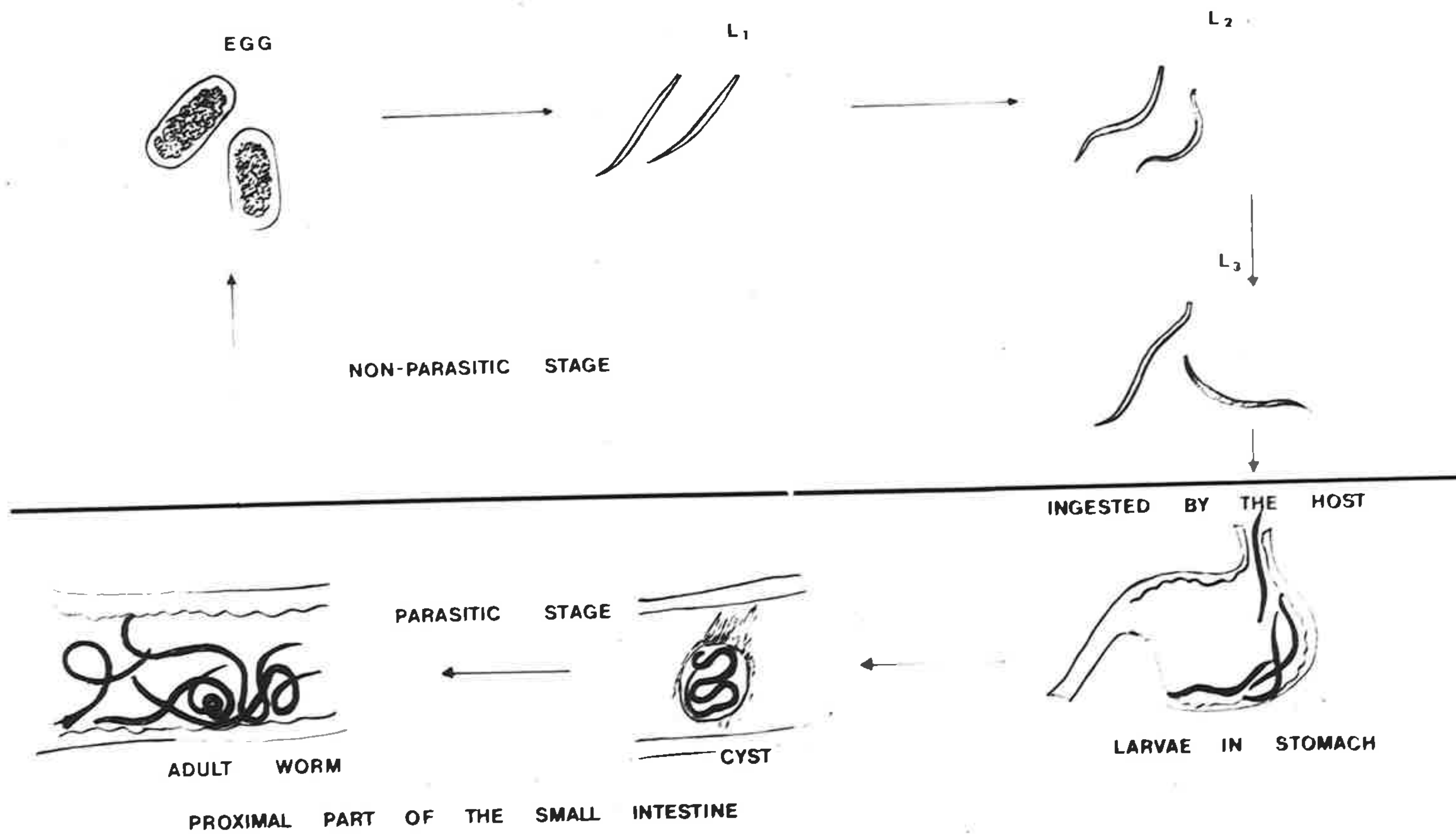


Figure 1.1

CHAPTER 2

MATERIALS AND METHODS

1. EXPERIMENTAL ANIMALS

In most experiments an outbred strain of Swiss White mice were used. However, some of the experiments, indicated in the text, involved the use of two inbred strains of mice; Balb/C and CBA and an F1 hybrid between an inbred C57 black strain and the Balb/C. Unless otherwise stated, all mice were used at 7-9 weeks of age.

2. MAINTENANCE OF THE PARASITE, *Nematospiroides dubius*

Third stage larvae of *N. dubius* were kindly supplied by Dr. R.I. Sommerville of the Zoology department, University of Adelaide, South Australia. The stock was maintained by infecting female Swiss White mice orally with about 300-400 larvae. The faeces of these infected mice were collected on the ninth day after inoculation by placing moist filter paper as a lining in the bottom of the cage (modified after Baker, 1954). The faecal pellets were mixed with distilled water and, after standing at room temperature for 15 min., passed through a fine 500 μ nylon mesh. After centrifuging the filtrate at 1500 g for 15 min. the supernatant was discarded and the sediment resuspended in approximately 10 ml of distilled water. Using a 10 ml pipette, the suspended sediment was streaked onto moist Whatman No. 41 filter paper lining a large petri dish (Figure 2.1). The dishes were incubated at 28°C and 100% humidity. Each day for a period of 15 min. the lids of the petri dishes were removed to allow aeration of the culture.

After 7 days the larvae were collected by rinsing the filter paper in the petri dishes with distilled water. By this time the larvae were in the third stage i.e. the infective stage. After three washings the larvae were suspended in distilled water and kept at 4°C until required. Third stage larvae may be maintained under these conditions for periods of up to one month and remain fully infective. The number of larvae in the culture was determined by placing a known volume of a suitable dilution onto a perspex scale, covering this with a cover slip and counting the parasite under a dissecting microscope.

2.1 Method used for exsheathing third stage larvae

Three to four thousand third stage larvae in 0.5 ml of distilled water were placed in a small bag made from dialysis tubing (Selbys, Visking, Chicago). Under ether anaesthesia, an incision was made in the abdomen of a normal Swiss White mouse and the bag inserted into the peritoneal cavity. The abdomen was closed and after 18 hours, the mouse killed by cervical dislocation and the bag recovered from the peritoneal cavity. Under these conditions more than 75% of the larvae were completely exsheathed whilst the remainder were partly so. The larvae recovered from the bag were washed three times in either 199 medium (Commonwealth Serum Laboratories, Melbourne) or Hank's balanced salt solution before being used to infect the mice (Weller, Enders, Robbins and Stoddard, 1952).

2.2 Implantation of the third stage larvae of *N. dubius* into the small intestine of mice

Laparotomy was performed on the anaesthetised mouse to expose the proximal part of the small intestine. Exsheathed larvae in 0.2 ml of medium were injected into the small intestine through a 23 gauge needle. Antibiotic powder was applied to the surgical wound before the abdominal wall was closed.

3. METHOD FOR IMMUNISING MICE AGAINST INFECTION AND ASSAY OF IMMUNITY

Mice were injected intravenously with one, two or three doses of 200 third stage larvae in 0.2 ml of physiological saline. Fourteen-day intervals separated each injection. Preliminary experiments had shown that after intravenous injection a few larvae were able to encyst in the wall of the small intestine and emerge as adult worms. Since assessment of immunity was based on cyst and adult worm counts, it was found necessary to treat the mice orally with carbon tetrachloride before challenge with a standard dose of third stage larvae in order to remove these adult worms (Panter, 1967). Treatment with carbon tetrachloride was given 10 days after the last immunising dose and the mice were challenged with infective larvae 5 days after this treatment. In some experiments mice were immunised orally as described by Van Zandt (1961) and Panter (1969a). Third stage larvae were introduced into the lower part of oesophagus using a tipped 19 gauge needle connected to a tuberculin syringe. The number of larvae administered by this route is mentioned in the text. In all experiments an equal number of non-

immunised mice served as controls, and were treated also with carbon tetrachloride as above. The number of encysted larvae and adult worms in the control group was compared with the number in the immunised group. The number of cysts were counted on the fifth or sixth day after challenge with the infective third stage larvae. The small intestine was removed from just below the pyloric sphincter to the ileo-caecal valve and pressed between two pieces of transparent perspex (Figure 2.2). The whole of the exposed intestine was then scanned under a dissecting microscope and the cysts counted. To determine the numbers of adult worms, mice were killed on the tenth day following infection. The small intestine was opened longitudinally and placed in a petri dish containing physiological saline. By warming the petri dish to 37°C, the adult worms became active, and could be recovered from the saline using a pasteur pipette. In this way the adult worms could be counted (Figure 2.3).

4. PREPARATION OF ISOTOPICALLY LABELLED EXSHEATHED THIRD STAGE LARVAE OF *Nematospiroides dubius*

Exsheathed larvae were washed several times with 199 medium containing 10% foetal calf serum and resuspended in the same to a concentration of 3,000 - 4,000 larvae/ml. Two hundred microcuries of ^{51}Cr in the form of sodium chromate (Radiochemical Centre, Amersham, England) were added to 2 ml of the suspension. The mixture was incubated at 28°C for two hr. After this period of incubation, the

larvae were washed four times with 199 medium and the amount of radioactivity associated with the larvae assayed in a Packard Liquid Scintillation Counter. In some other experiments the exsheathed larvae were labelled with ^{32}P. The technique was essentially the same, 150 microcuries of ^{32}P as orthophosphate being added to 6,000 - 8,000 exsheathed larvae. After overnight incubation at 28°C the larvae were washed four times with fresh 199 medium containing 100 U of penicillin and 100 μg of streptomycin/ml and the amount of ^{32}P associated with the larvae measured in a Nuclear Chicago End Window Counter.

5. COLLECTION AND PREPARATION OF ANTIGENIC MATERIAL FROM *N. dubius*

Adult worms were collected from the small intestine after oral challenge with third stage larvae. After killing the mouse by cervical dislocation, the small intestine was removed, opened longitudinally and placed in physiological saline at 37°C in a modified Bauman's apparatus (Barth, Jarrett and Urquhart, 1966). The adult worms moved down to the bottom of the funnel and finally accumulated in the flask. Worms taken at this time contained few eggs and were a suitable source of antigenic material. Antigenic extracts were prepared from third stage larvae and adult worms by techniques similar to those used by Ogilvie (1964), Panter (1967) and Cathy (1969). After repeated washings with physiological saline, both larvae and adult worms were resuspended in this solution to give a concentration of 250 mg wet weight/ml. After cooling in ice, 4 ml of the suspension was homogenized for 30 sec. in a glass tissue grinder

using an electrical driven pestle. During the whole of this procedure the vessel was kept in ice. The resulting homogenate was then subjected to ultrasound (MSE, Thomas Optical & Scientific Co. Pty. Ltd.) at 4°C for 25 min., and finally centrifuged at 4,000 g for 30 min. The supernatant resulting from this centrifugation was kept at -20°C until required.

6. PREPARATION OF SPLEEN AND LYMPH NODE CELLS FROM THE MOUSE

6.1 Preparation of spleen cells:

Spleens taken from either normal or immunised mice were cut into several small pieces and gently pressed with a glass rod through a stainless steel sieve (250 μ mesh) into a petri dish containing ice cold 199 medium with 10% foetal calf serum to which had been added 3 I.U./ml of preservative-free heparin, 100 U/ml penicillin and 100 μg/ml streptomycin. Using a pasteur pipette the suspension was gently pipetted up and down several times then allowed to stand for a few minutes to enable the larger clumps of cells to settle out. The medium was then carefully removed and centrifuged at 45 g for 30 sec. The supernatant resulting from this procedure eventually contained single cells. These cells were then further washed three times with ice cold 199 medium as above by centrifugation at 60 g for 15 min. at 4°C. After washing, the cells were resuspended in the 199 medium to give a final concentration of 10^8 cells/ml. More than 90% of these cells were viable as judged by trypan blue exclusion.

6.2 Preparation of lymph node cells

Lymph node cells were prepared from the brachial, popliteal cervical, internal axillary and mesenteric lymph nodes by the method described above.

6.3 Method for collecting thoracic duct lymphocytes

The method was that described by Boak and Woodruff (1965) and Miller and Mitchell (1968). Mice were given 1 ml of cream orally using a blunt, curved 19 gauge needle mounted on a 1 ml syringe. One hr after feeding the mice were anaesthetised by giving intraperitoneally 1.5 mg of sodium pentobarbitone/20 gm body weight. An area was shaved in the lumbar region and an incision made along the lateral border of the left quadratus lumorum muscle in order to expose the left kidney and the adjacent organs. The wound was then packed with sterile gauze and the kidney deflected away from the lumbar muscles. Using blunt dissection, the retro-peritoneal tissue over the descending aorta was removed to expose the thoracic duct. A boak cannula (Portex Ltd., England) filled with physiological saline containing 50 I.U. heparin/ml was then inserted into the thoracic duct at a point just above the renal artery. A small drop of COAPT adhesive (Ethicon, Hamberg, Germany) was applied to the point of entry of the cannula to seal the wound and hold the cannula in place. The muscles and skin layers were closed separately using interrupted sutures in a manner which allowed the cannula to protrude in the lumbar region. The mice were then strapped

to the apparatus illustrated in Figure 2.4 in such a way that they could run on the drums. The mice were provided with food and dextrose saline *ad lib*. Recovery was usually rapid and the movement of the mice facilitated the flow of the lymph which was collected in ice cold 199 medium containing 5 I.U./ml heparin. The lymphocytes were washed and made to a final concentration of 5 x 10^7 cells/ml. The cells treated in this manner were more than 90% viable as judged by exclusion of trypan blue.

6.4 Preparation of monolayers of mouse peritoneal macrophages

Peritoneal macrophages were harvested from the peritoneal cavity by the method previously described by Rowley and Whitby (1955). The cells were harvested in 199 medium or Hank's balanced salt solution containing 5 I.U./ml heparin and added to Leighton tubes. The tubes were incubated at 37°C. for 45 min. to allow the cells to settle and adhere to the glass. After this settling period the medium was removed and fresh heparin free 199 medium added. The monolayers were then ready for use in the experiments described in the text.

7. PREPARATION OF ANTISERUM AGAINST *Nematospiroides dubius* INFECTION

Mice were injected intravenously on three occasions with 200 living third stage larvae. Fourteen day intervals separated each injection. Seven days after the last injection the mice were bled from the retro-orbital plexus. The blood was allowed to clot at room

temperature, and the serum removed and stored at -20°C until required.

8. ASSAY OF SPECIFIC ANTIBODY TO ANTIGENS FROM EITHER LARVAL OR ADULT WORMS USING AN INDIRECT HAEMAGGLUTINATION TEST

The procedure of Avrameas *et al.* (1969) was followed. Antigens from larvae or adult worms were coupled to erythrocytes by the following procedure. Protein antigens (30 mg) in 10 ml phosphate buffer saline (PBS) pH 6.4 were added with 0.1 ml washed, packed sheep red blood cells. While the mixture was gently stirring, 0.6 ml of a 2.5% solution of glutaraldehyde was added. The stirring was continued for one hr at room temperature. The cells were then washed three times in PBS at pH 7.2 containing 1% heat-inactivated normal rabbit serum by centrifugation at 1200 g for 10 min. Finally they were resuspended in the same buffer to a final concentration of 2% at 4°C until required.

Two-fold dilutions of immune serum were prepared in saline using the microtitre technique (Microtitre Instruction Manual, 1969). An equal volume of the sensitized cells was added to each well. The haemagglutinating titre was read after 1 hr at room temperature. One haemagglutinating unit was taken as the highest dilution of serum which gave complete agglutination of the sensitized sheep erythrocytes.

9. STATISTICAL ANALYSIS

Most of the results were analysed for significance by using the student's "t" test. The F distribution was used on some occasions

(Finney, 1952; Fisher, 1970). When appropriate, the non-parametric Mann-Whitney U test was used (Mann and Whitney, 1947 and the extended table by Auble (1953); Siegel (1956) and Rohlf and Sokal (1969). A P value of $P = < 0.05$ was taken as being significant.

LD50's were estimated by the method of Miller and Tainter (1944).

10. TISSUE CULTURE MEDIA

a. 199 medium

The medium was purchased from the Commonwealth Serum Laboratories, Melbourne, Australia, without antibiotics at 10 x concentration.

b. Hank's balanced salt solution

The medium was used as described by Weller *et al.*, (1952). It was buffered to the desired pH with 2.8% sodium bicarbonate. Before use the medium was gassed with 5% CO_2 in air.

FIGURE 2.1

Streaks of mouse faeces used for the *in vitro* culture of the larval stage of *N. dubius*.

Figure 2.1

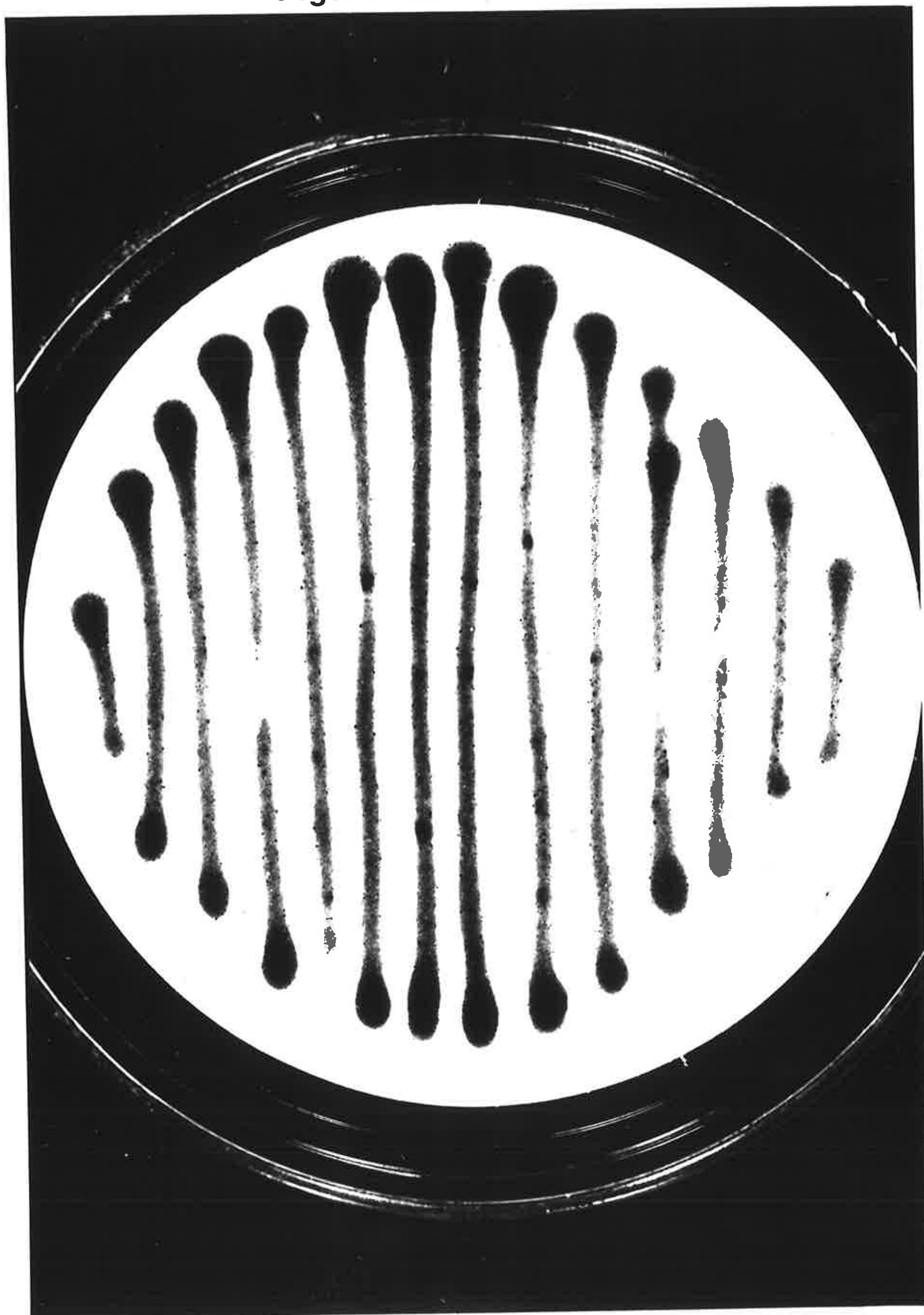


FIGURE 2.2

The small intestine of the mouse showing the encysted form of Nematospiroides dubius.

C = Cyst

Figure 2.2

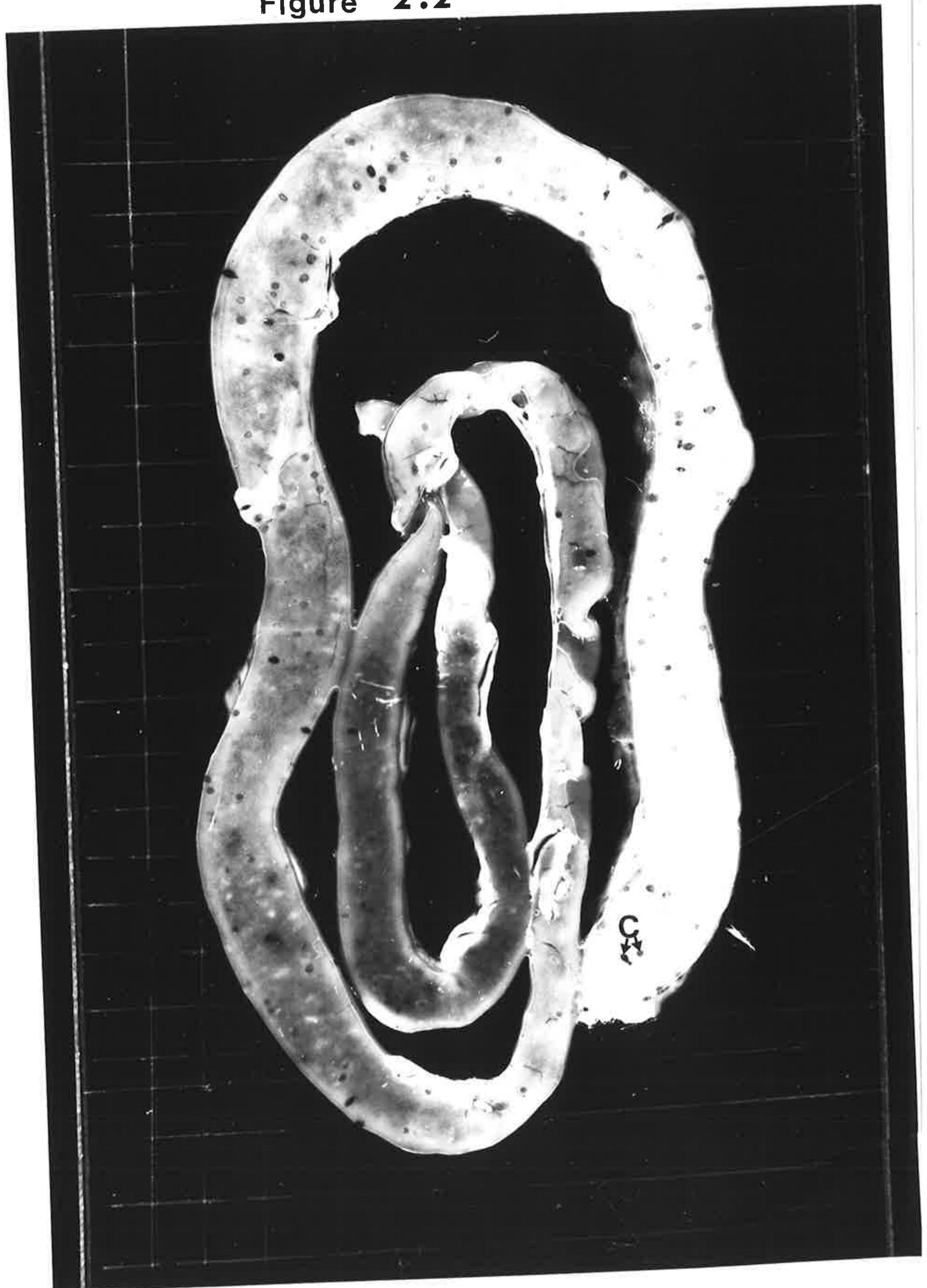


FIGURE 2.3

Petri dish containing the opened small intestine of an infected mouse showing the adult stage of *N. dubius*.

I = Intestine

W = Adult stage

Figure 2.3

FIGURE 2.4

Apparatus used for collecting thoracic duct lymphocytes from adult mice.

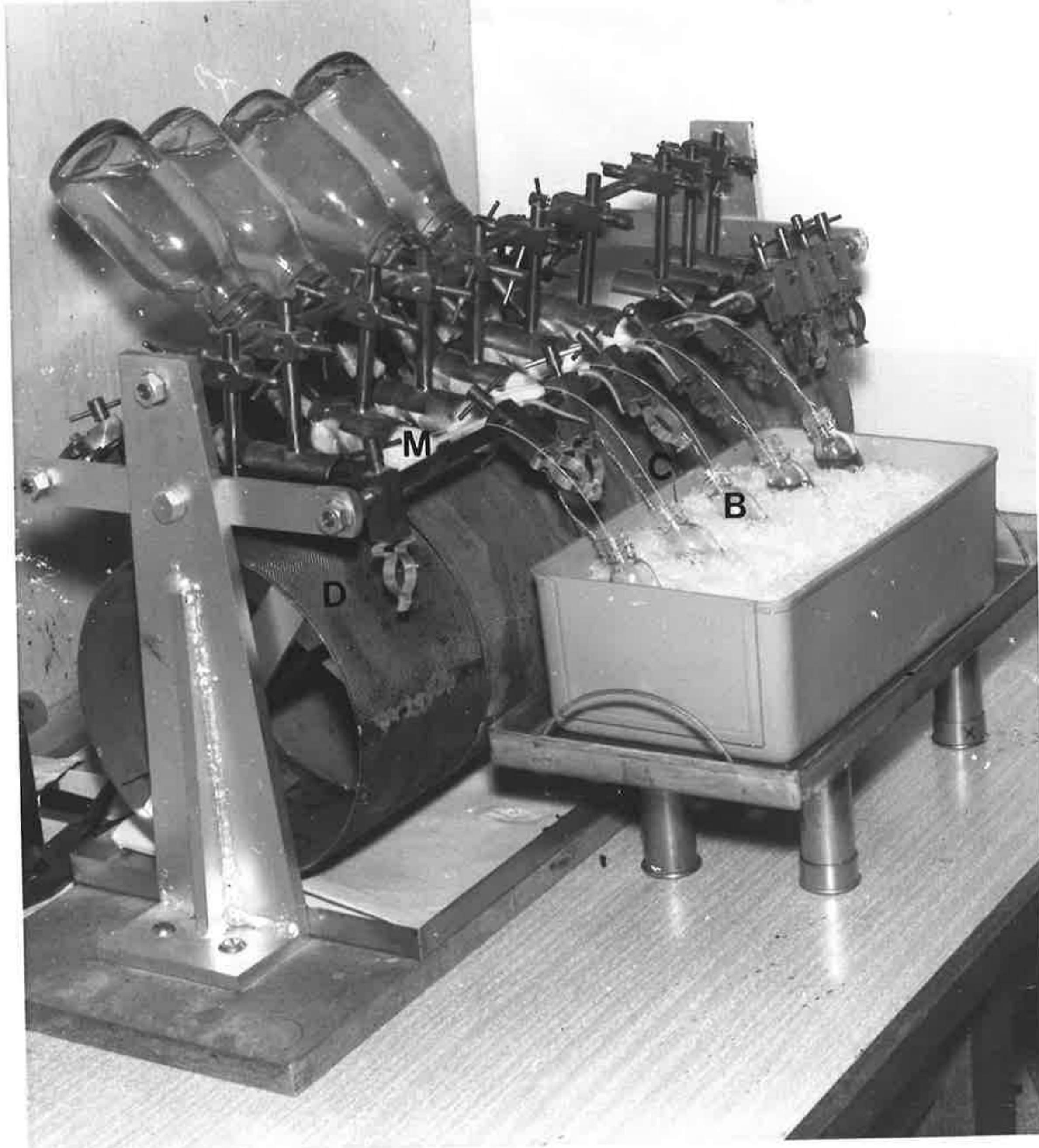
B = Bottle containing 199 medium in ice bath

C = Cannula

D = Revolving drums

M = Mouse

Figure 2.4



CHAPTER 3

THE HOST-PARASITE RELATIONSHIP OF MICE AND *Nematospiroides dubius*

1. INTRODUCTION

Before investigating methods of inducing immunity in mice to *N. dubius* infections, it was necessary to determine the susceptibility of the mouse strains to be used in this investigation to this infection.

Initial studies were carried out in Swiss White mice using animals of different ages and challenged with different numbers of infective third stage larvae.

2. THE INFECTIVITY OF *Nematospiroides dubius* FOR SWISS WHITE MICE OF DIFFERENT AGES

Groups of 15 of an outbred strain of Swiss White mice aged 1, 4, 15, 21 and 49 days were challenged orally with 80 third stage infective larvae. An equal number of mice from each group were killed on day 6 and 10 to determine the number of cysts and adult worms respectively. The results of these experiments given as the percentage recovery of the challenge dose indicate that one-day-old mice are less susceptible to this infection than older ones. The susceptibility increased with age and reached a maximum at about 21 days of age (Fig. 3.1).

In view of these results it was clear that seven-week-old mice would be of a suitable age for measuring changes in resistance to

FIGURE 3.1

The percentage recovery of cysts and adult worms from 1-, 4-, 15-, 21- and 49-day old mice after challenging with 80 third stage larvae of N. dubius orally.

a, f = 1 day old mice

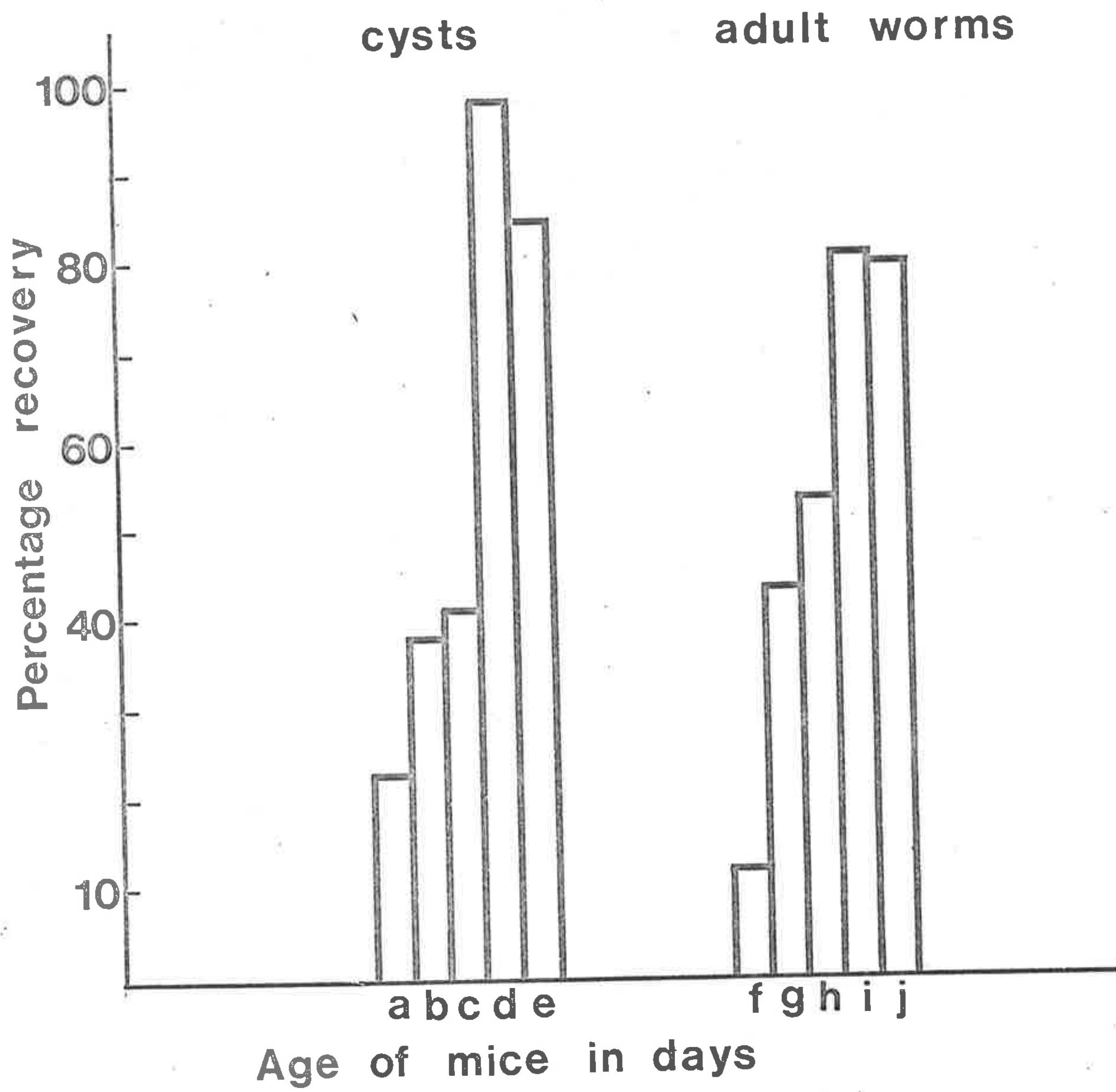
b, g = 4 day old mice

c, h = 15 day old mice

d, i = 21 day old mice

e, j = 49 day old mice

Figure 3.1



infection after various treatments. However it seemed important to establish that the susceptibility of the animal as measured by cyst and adult worm recovery was or was not related to the number of larvae used in the challenge dose.

3. RECOVERY OF CYSTS AND ADULT WORMS FROM SWISS WHITE MICE CHALLENGED WITH DIFFERENT DOSES OF INFECTIVE LARVAE

In the experiment 48 Swiss White mice aged seven-weeks were divided into two equal groups. Group 1 was infected orally with 45 third stage larvae whilst group 2 was infected with 192 larvae. Mice were killed on day 6 to determine the numbers of cysts and on day 10 to count the number of adult worms. Data from these experiments are given in Table 3.1. It may be seen that the percentage recovery of either cysts or adult worms was independent of the challenge dose.

4. RECOVERY OF CYSTS AND ADULT WORMS IN BALB/C MICE

Since Balb/C mice were used in some of the experiments reported later in this thesis it was necessary to show that in normal animals given a certain dose of infective larvae, the recovery of cysts and adult worms was similar to that in the outbred Swiss White strain. Ten female Balb/C and 10 female Swiss White mice were challenged with 170 infective larvae. They were killed at similar time intervals as above and the cyst and worm burden counted. Results given in Table 3.2 show that in terms of cyst and

<u>TABLE 3.1</u> Cysts and adult worms recovered from Swiss White mice which had received 45 and 192 third stage larvae of <u>N. dubius</u> orally.

	Cyst Number		Adult worm Number	
	Group 1*	Group 2	Group 3	Group 4
	31	164	43	161
	39	150	34	145
	47	169	31	163
	47	180	29	151
	42	177	32	169
	30	153	46	182
	50	179	33	153
	45	189	40	181
	42	198	36	178
	51	169	36	131
	38	172	33	174
			39	142
Mean	42.0	172.7	36.0	160.8
S.d. of mean	7.0	14.2	5.1	16.6
Percentage recovery	93.3	89.9	80.0	83.8

* Group 1 and 3 dosed with 45 third stage larvae
Group 2 and 4 dosed with 192 third stage larvae

TABLE 3.2 Cyst and adult worm recoveries from Balb/C and Swiss White mice which received 170 third stage larvae of *N. dubius*/mouse orally.

	Cyst numbers		Adult worm numbers	
	Balb/C	Swiss	Balb/C	Swiss
	173	148	159	128
	167	166	147	146
	171	156	141	121
	169	156	135	137
	168	168	140	115
Mean ± s.d.	169 ± 2.4	158.8 ± 8.2	143.6 ± 9.3	129.4 ± 12.4
Percentage recovery	99.7	93.4	84.5	76.1

adult worm recovery Balb/C mice were as susceptible as Swiss White mice to this particular infection.

In view of these results it seemed pertinent to determine the LD_{50} in the various strains of mice in order to choose a suitable dose of larvae to challenge immunised animals.

5. THE LD_{50} IN VARIOUS STRAINS OF MICE

Preliminary experiments indicated that despite the fact that Balb/C mice were as susceptible as Swiss White animals to the infection determined as above, they were unable to carry as heavy a worm burden. A significant number of the Balb/C strain died within thirty days of challenge with only 200 larvae. Thus in order to determine the LD_{50}, the various strains of mice were challenged as follows. Male Swiss White mice divided into groups of 10 were given 300, 400, 500, 600, 700 and 800 third stage larvae orally. An equal number of females were treated in a similar fashion. Mice of the Balb/C strain in groups of 10 were dosed with 50, 100, 200, 300 and 400 larvae, whilst male F1 hybrids (C57BL x Balb/C) received 200, 300, 400, 500 and 600 third stage larvae. Deaths were recorded over a period of thirty days. The results shown in Tables 3.3, 3.4, 3.5, 3.6 and Figs. 3.2. and 3.3 indicate that the Swiss White mice were more resistant than the Balb/C strain. The LD_{50} being 500 larvae in the former case but only 177 in the latter. In contrast the F1 hybrid mice were almost as resistant as the outbred Swiss White mice, the

TABLE 3.3 Mortality in male Swiss White mice infected with *N. dubius* third stage larvae of different doses

Dose of larvae	Day after infection																							Total % Mortality	Probits
	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	30		
	% Cumulative Mortality																								
300					10																	20		20	4.16
400									10		30						40							40	4.75
500					10	20					40													40	4.75
600									10	30			40			50		60						60	5.25
700					10	20		30	50		60	80												80	5.84
800	30	70	90	100																				100	8.72

TABLE 3.4 Mortality in female Swiss White mice infected with *N. dubius* third stage larvae of different doses

Dose of larvae	Day after infection																				Total % Mortality	Probits
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	30		
	% Cumulative Mortality																					
300																			10		10	3.72
400						10				20		30					40				40	4.75
500											10			30					40		40	4.75
600				10	20										30		40	50			50	5.00
700									10	20	30	40	50	70		80					80	5.84
800	10	80	90	100																	100	8.72

TABLE 3.5 Mortality in F1 (C57BL x Balb/C) mice infected with *N. dubius* third stage larvae of different doses

Dose of larvae	Day after infection																		Total % Mortality	Probits
	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	30		
			% Cumulative Mortality																	
200																10			10	3.72
300															10		20		20	4.16
400												20				40			40	4.75
500					20			40		50		60	80						80	5.84
600		60	80	100															100	8.82

TABLE 3.6 Mortality in Balb/C mice infected with *N. dubius* third stage larvae of different doses

Dose of larvae	Day after infection												Total % Mortality	Probits
	15	16	17	18	19	20	21	22	23	24	25	30		
	% Cumulative Mortality													
50						10							10	3.72
100				20	30			40					40	4.75
200			20	30		50							50	5.00
300		20		40	50	70	80						80	5.84
400			20		50	80	90		100				100	8.82

FIGURE 3.2

The LD50 of the third stage larvae of N. dubius for Swiss White mice.

A = Male mice

B = Female mice

Figure 3.2

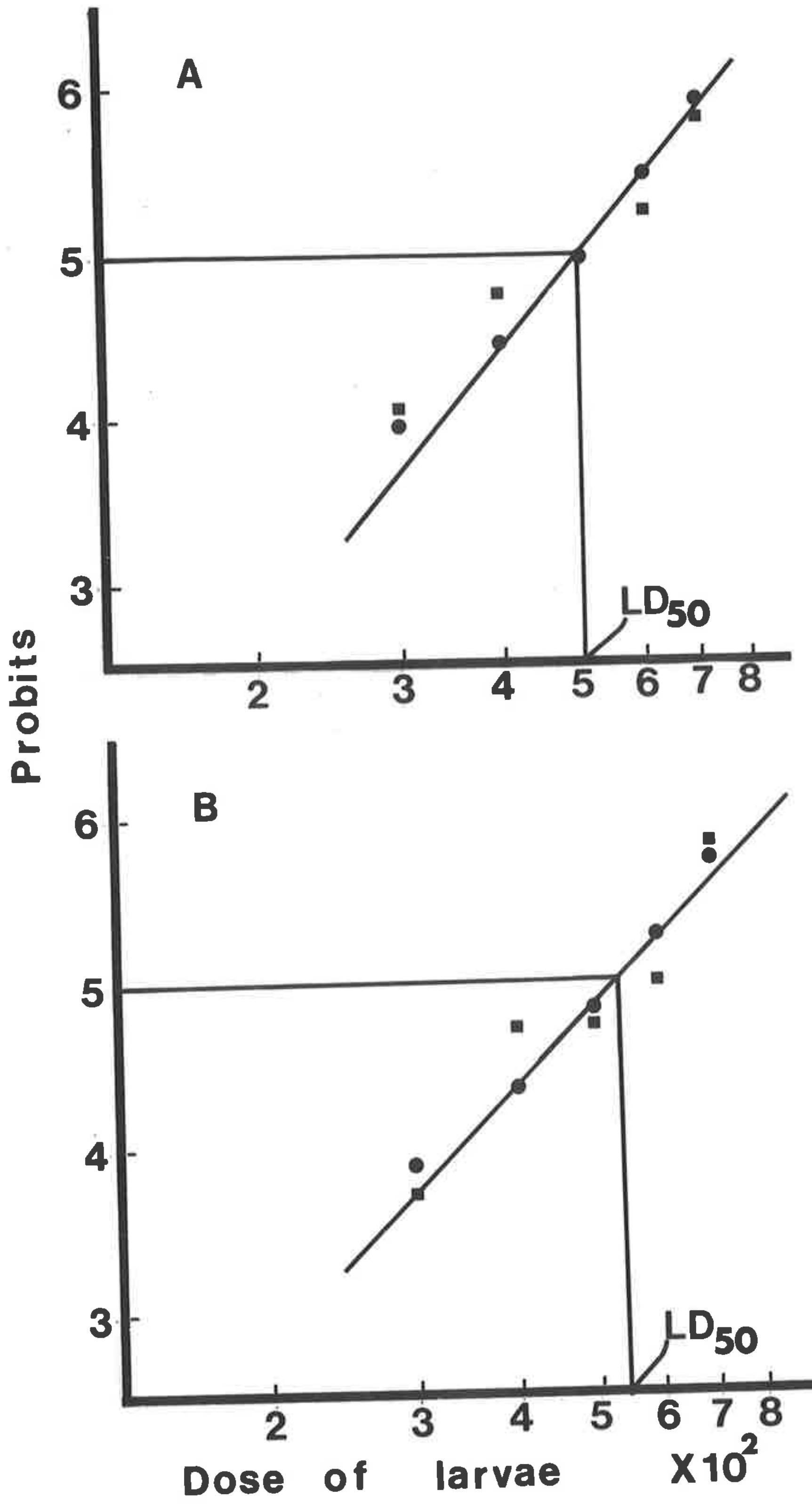


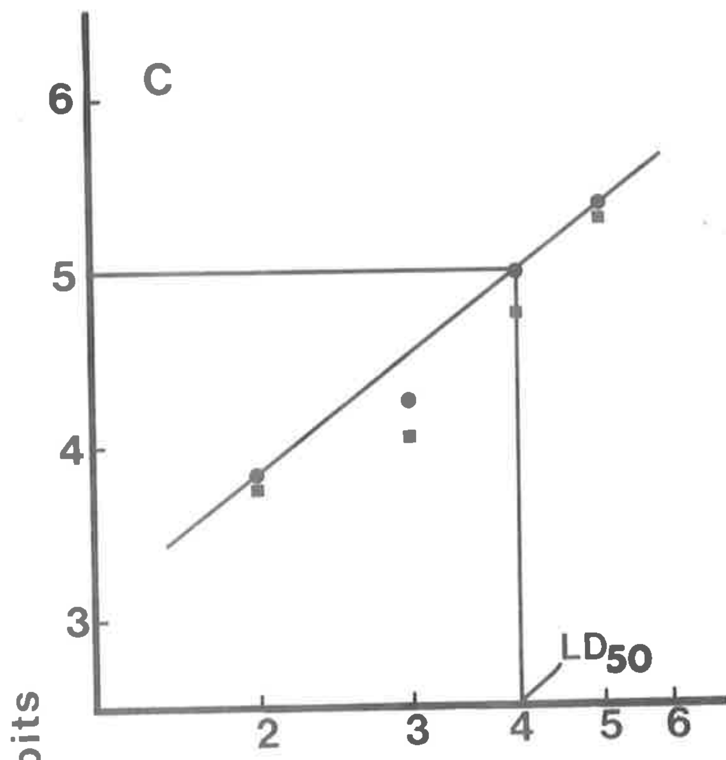
FIGURE 3.3

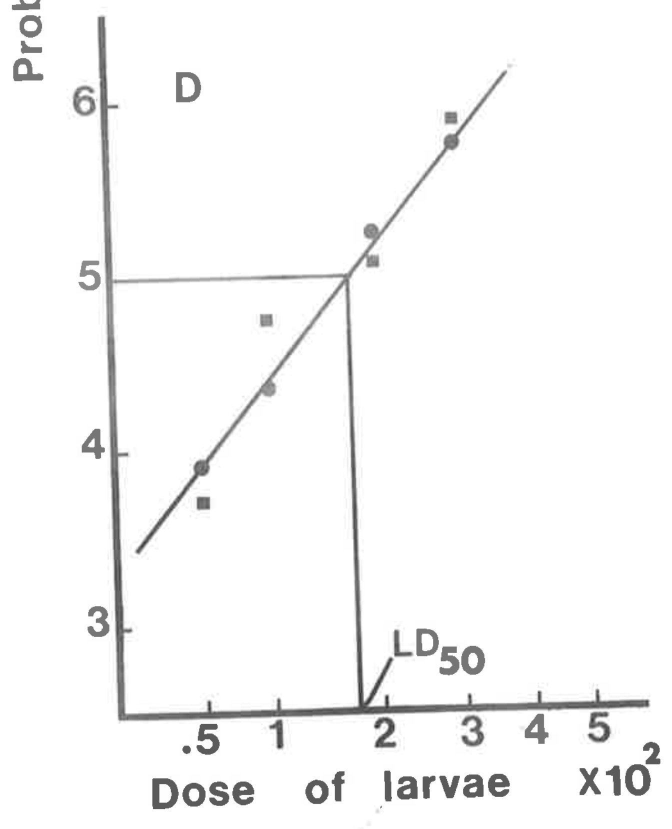
The LD50 of the third stage larvae of *N. dubius* for F1 hybrid (C57BL x BALB/C) and Balb/C mice.

C = F1 hybrid mice

D = Balb/C mice

Figure 3.3





LD_{50} being 400 larvae. There was no significant difference between males and females of the Swiss White strain in terms of susceptibility to this infection. The LD_{50} was calculated using the method of Miller and Tainter (1944), the best fitting straight line being determined by the method of least squares (Yamane 1973).

6. HISTOPATHOGENIC CHANGES IN THE SMALL INTESTINE AFTER INFECTION WITH THIRD STAGE LARVAE

Eight female Swiss White mice were challenged orally with 200 infective larvae. On day 7 four of the mice were killed, the proximal part of the small intestine removed and fixed in Bouins reagent prior to sectioning. After imbedding in paraffin, 6 μ sections were cut through the encysted larvae, and the sections stained with haematoxylin-eosin. In some cases a direct smear was made of the cyst contents, after rupturing the cyst with a sharp needle. This was performed under a dissecting microscope.

Sections indicated some haemorrhage during the penetration of the larvae into the intestinal wall. Erythrocytes were present in the cyst fluid as were a few granulocytes. The muscle layers in the region of the cyst were oedematous and the small blood vessels in the same area were damaged. Thickening of the walls of the blood vessels was also apparent. Localised inflammatory changes were apparent in the area and both sections and smears revealed an infiltration of granulocytes and mononuclear cells (see Figure 3.4).

On day fifteen the remaining four mice were killed. By this time the encysted larvae had developed into adult worms and were present in the gut. Examination of section of the empty cysts revealed the presence of cells that consisted mainly of macrophages. A few giant cells were also found. The cyst was surrounded by fibrous tissue cells and by this time the muscle layers were no longer oedematous (see Figure 3.5).

7. CONCLUSIONS

The results given in this chapter show that new born mice of the Swiss-White strain are more resistant to infection than are adult mice. However, at the time of weaning i.e. three weeks, the mice were as susceptible as adult animals. All the strains of mice studied were equally susceptible to infection as indicated by the recovery of cysts and adult worms, although data obtained from LD_{50} experiments show that the Balb/C strains are unable to carry as heavy a worm burden as the Swiss-White.

FIGURE 3.4

T.S. of the small intestine from a normal mouse showing the histophatological changes 7 days after a primary infection. Note the cellular infiltration of macrophages, lymphocytes and polymorphonuclear cells around the cyst (C) containing the larval stage (L) of N. dubius. Compare with Figure 4.1.

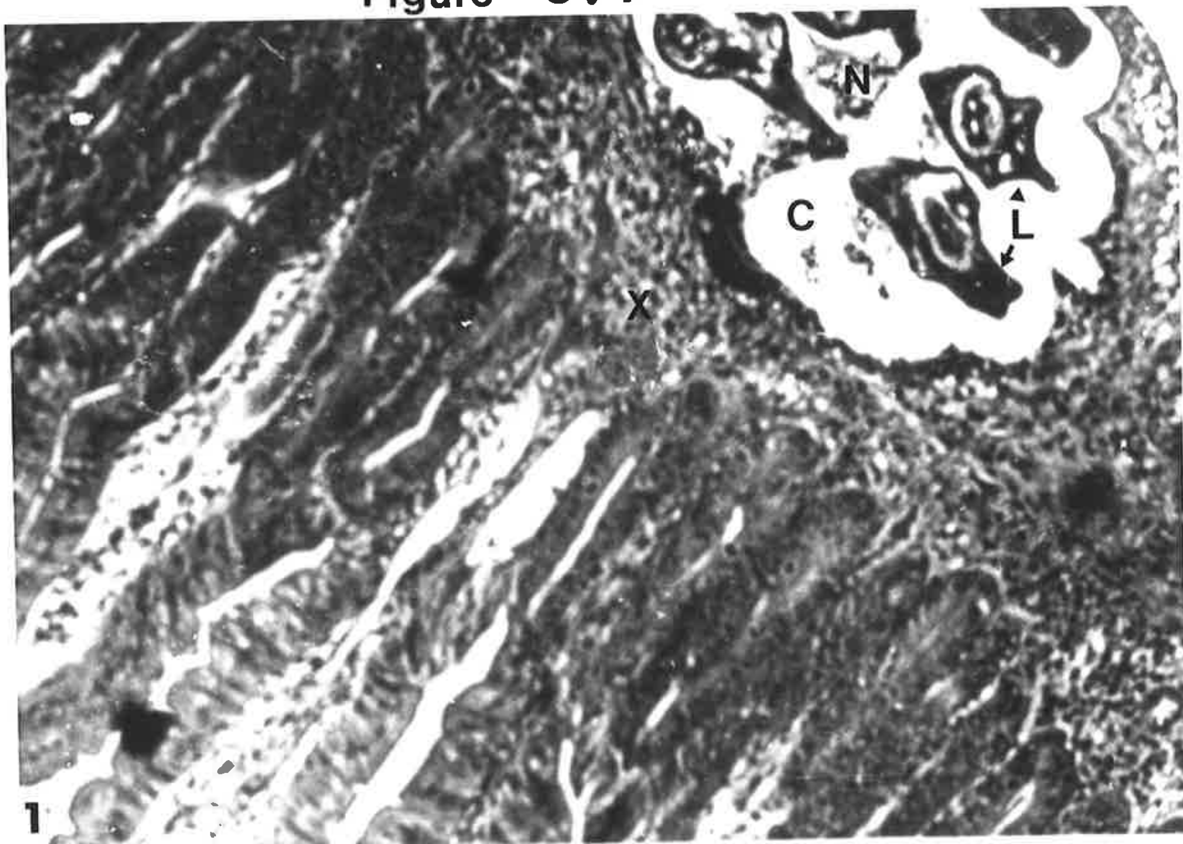
LY = Lymphocyte

M = Macrophage

N = Polymorphonuclear cell

1. Haematoxylin-eosin 170X
2. Haematoxylin-eosin 1100X

Figure 3.4



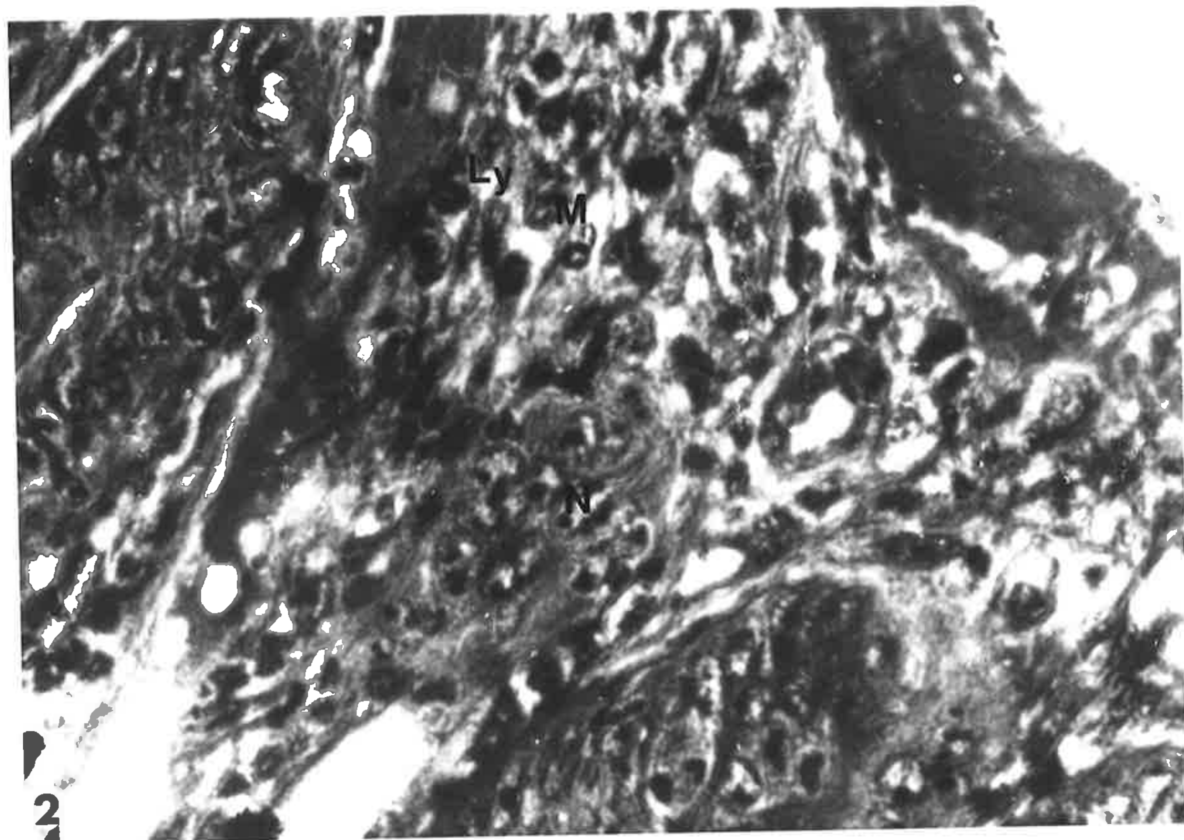


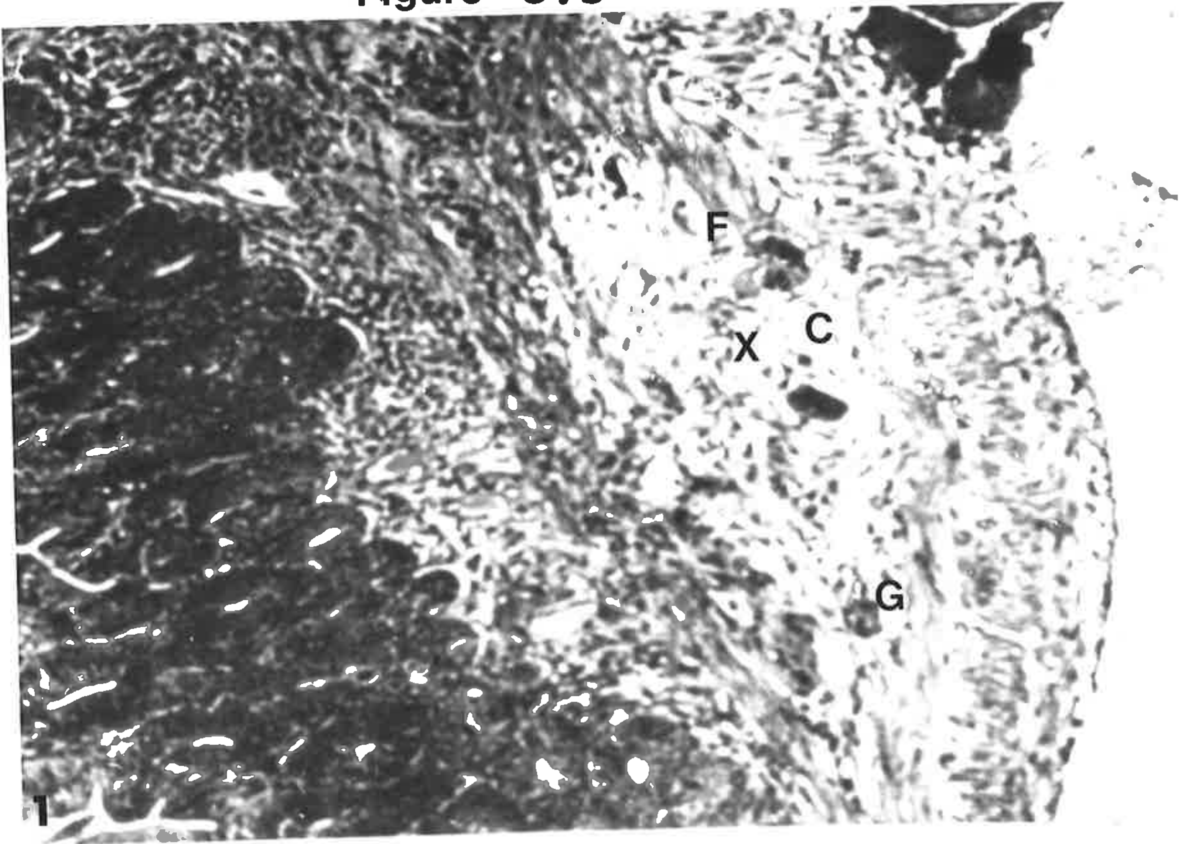
FIGURE 3.5

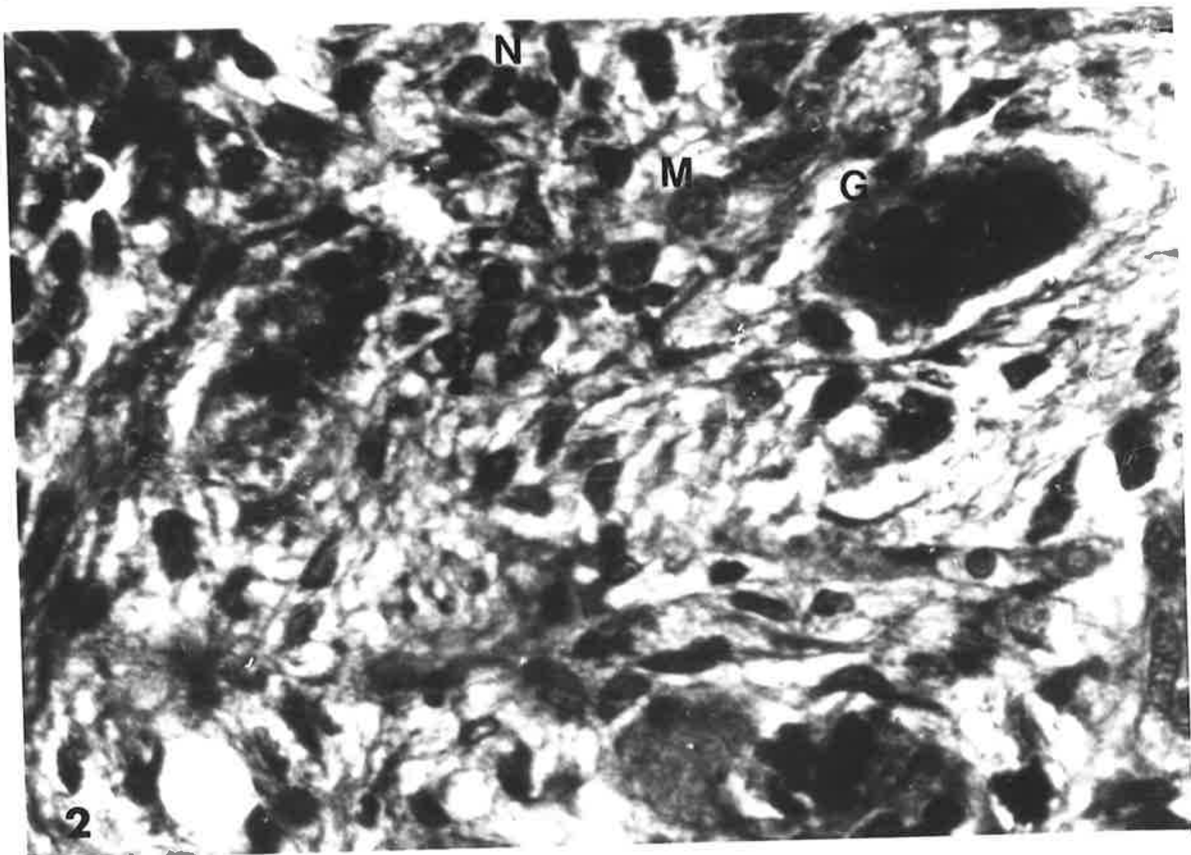
T.S. of the small intestine from a normal mouse showing the histopathological changes 15 days after a primary infection. Note the cellular infiltration composed of macrophages, lymphocytes and polymorphonuclear cells. Fibrosis (F) has occurred around the cyst (C). Compare with Figure 4.2.

G = Giant cell
F = Fibrous tissue
LY = Lymphocyte
M = Macrophage
N = Polymorphonuclear cell

1. Haematoxylin-eosin 170X
2. Haematoxylin-eosin 1100X

Figure 3.5





CHAPTER 4

INDUCTION OF IMMUNITY IN MICE TO INFECTION WITH *Nematospiroides dubius*

PART 1: INDUCTION OF RESISTANCE BY NON-SPECIFIC MEANS

1. INTRODUCTION

Numerous studies have shown that prior treatment of mice with lipopolysaccharides from gram-negative bacteria will increase their resistance to a variety of bacterial infections (e.g. Condie, Zak and Good, 1955; Rowley, 1955; and Boehme and Dubos, 1958).

This increase in resistance is due in part to a change in the activity of the phagocytic cells as measured by an increase in their rate of metabolism, enhanced phagocytosis in the presence of limiting amounts of antibody when compared with cells taken from normal animals, and an increase in their content of lysozomal enzymes (Benacerraf and Sebestyen, 1957; Biozzi, Benacerraf and Halpern, 1955; Boehme and Dubos, 1958; Auzins and Rowley, 1962; Coppel and Youmans, 1969 and McKay, Jenkin and Tyson, 1973). Concomitant with an increase in the activity of the phagocytic cells, there is an increase in the total amount of circulating specific antibodies that the animal was synthesising prior to stimulation (Thorbecke and Benacerraf, 1962; Stuart and Davidson, 1964; Franzl and McMaster, 1968a,b). These two changes may express themselves as an increase in resistance to infection with various parasites: viral infections (Ginsberg, Goebel and Horsfall, 1948; Wagner, Snyder, Hook and Luttrell, 1959), fungal infections (Hasenclever and Mitchell, 1962;

1963), and protozoal infections (Styles, 1965; Foris, 1970). More recently other workers have shown that mice pretreated with B.C.G. *Corynebacterium parvum*, or a rough strain of *Salmonella enteritidis* (*S. enteritidis* IIRX) show increased resistance to various tumours. This increased resistance appears to be related to the production of 'activated' macrophages as a result of the above treatments. This 'activation' is measured as a direct cytotoxic effect which these cells have on the various tumour lines (Old, Clarke and Benacerraf, 1959; Fisher, Grace and Mannick, 1970; Hardy and Kotlarski, 1971; Alexander, Evans and Grant, 1972; Ashley and Hardy, 1973).

Because resistance to metazoan parasites may involve such immune mechanisms which are similar to those concerned in resistance to tumours and other foreign tissue transplants, it seemed pertinent to attempt to alter the susceptibility of mice to infection with *Nematospiroides dubius* by such non-specific treatments.

2. CHANGES IN SUSCEPTIBILITY TO INFECTION FOLLOWING INJECTION OF S. ENTERITIDIS IIRX

Groups of 12 female Swiss White mice previously injected intraperitoneally with *S. enteritidis* IIRX such that each mouse received 1×10^5 bacteria, were challenged orally with 350 third stage larvae. The experiment was arranged such that all the mice plus 36 controls received the larvae on the same day but the mice had carried the *S. enteritidis* for varying periods of time, as indicated in Tables 4.1 and

TABLE 4.1 Cyst recovery from mice which had received 1×10^5 IIRX intraperitoneally before challenge orally with 350 infective larvae.

	Length of time mice infected with IIRX/days					
	2	4	7	14	21	Control
	Number of cysts recovered					
Mean	222.16	233.66	212.66	191.50	206.00	258.12
Range	206-239	195-250	190.244	165-237	160-232	201-319
S.D.	10.9	25.8	21.08	29.18	27.86	39.96

P value "U" test

2 days vs control $P < 0.05$

4 days vs control $P > 0.05$

7 days vs control $P < 0.005$

14 days vs control $P < 0.001$

21 days vs control $P < 0.025$

TABLE 4.2 Adult worm recovery from mice which received 1 x 10^5 IIRX intraperitoneally before challenge with 350 infective larvae orally.

	Length of time mice infected with IIRX/days					
	2	4	7	14	21	Control
	Number of worms recovered					
Mean	198.33	163.17	146.50	168.16	156.60	192.88
Range	162-239	124-194	119-163	158-189	142-205	141-265
S.D.	33.10	24.94	18.76	13.86	28.54	28.48

P value "U" test

2 days vs control $P > 0.05$

4 days vs control $P < 0.025$

7 days vs control $P < 0.001$

14 days vs control $P < 0.005$

21 days vs control $P < 0.025$

and 4.2. Fifty percent of the animals were killed on day 6 to ascertain the number of cysts and the remainder on day 10 to determine the number of adult worms. Data expressed in Table 4.1 and 4.2 show that 14 days after the injection with S. enteritidis IIRX there was a significant but not marked increase in resistance to this infection.

3. CHANGES IN SUSCEPTIBILITY TO INFECTION AFTER INJECTION OF LIPOPOLYSACCHARIDE

Two groups of 12 male Swiss White mice eight weeks of age that had been injected intraperitoneally with 25 μg of a lipopolysaccharide obtained from Salmonella typhimurium (C5.) were challenged orally with 180 third stage larvae. The experiment was arranged such that all the mice were challenged with larvae on the same day, but the groups varied according to the length of time between treatment with lipopolysaccharide and challenge with larvae. All mice including 12 controls were killed on day 6 and the number of cysts counted. Results given in Table 4.3 show that the increase in resistance, though slight, was significant, but the duration was short and had disappeared by day 7. Similar results as regards the duration of immunity have been reported by others for bacterial infections.

PART 2 INDUCTION OF IMMUNITY TO Nematospiroides dubius INFECTION BY SPECIFIC IMMUNISATION

4. INTRODUCTION

Previous data in the literature had shown that mice that had

TABLE 4.3 Cyst recovery from mice treated with lipopolysaccharide and challenged with 180 infective larvae orally at 24, 48, 72, 96 hr and on day 7 following the injection of endotoxin.

	Time (hr and day) challenge after C_5 endotoxin injection					
	24	48	72	96	7	Control
	Number of cysts recovered					
Mean	116.0	126.2	120.2	121.9	156.0	158.7
Range	74-162	94-156	101-130	94-145	120-187	128-190
S.D.	24.9	17.5	9.4	15.4	19.7	15.0

P value "U" test

24 hr vs control $P < 0.001$

48 hr vs control $P < 0.001$

72 hr vs control $P < 0.001$

96 hr vs control $P < 0.001$

7 days vs control $P > 0.05$

been exposed to a primary infection with the parasite were resistant to further infection. However the degree of resistance was related to the number of subsequent exposures following the primary infection as demonstrated by the 23% of adult worm recovered in immune mice compared to 94% in controls (Panter, 1967). In view of the time taken to develop a good immunity after infection by the oral route it seemed desirable to seek other routes of immunisation.

5. CHANGES IN RESISTANCE TO INFECTION AFTER INTRAVENOUS INJECTION OF LIVING THIRD STAGE LARVAE

Before examining the possible immunising action of intravenous injections of living third stage larvae it was important to determine whether or not the immunising dose could establish itself in the gut after this abnormal route of infection.

Forty-two female Swiss White mice were injected intravenously with 220 larvae in 0.2 mls of saline. Six days later 21 mice were killed to determine the number of cysts in the intestine and the remainder on day 12 to determine the number of adult worms. The results presented in Table 4.4 show that contrary to expectations a high percentage of the injected larvae were able to establish themselves in their normal environment.

In order to assess any immunity that might result from intravenous injection of third stage larvae the mice were treated orally in the

TABLE 4.4 The recovery of cysts and adult worms from Swiss White mice immunised intravenously with 200 third stage infective larvae of *N. dubius*.

	Cyst numbers	Adult worm numbers
	107	126
	115	63
	110	186
	142	90
	93	73
	115	168
	90	187
	70	133
	149	147
	139	179
	224	156
	120	197
	190	128
	176	122
	115	197
	136	193
	125	190
	105	126
	78	73
	170	86
	117	156
Mean	127.9	141.7
S.D. of mean	37.7	44.8
Percentage recovery	58.1	64.4

following experiments with 0.2 ml of 25% carbon tetrachloride in 0.3% agar on day 9 after each immunising injection. Such treatment resulted in eliminating most of the adult worms. Control mice were also treated with carbon tetrachloride.

Swiss White mice were divided into three groups. Group 1 received 200 larvae intravenously, and, after the above treatment on day 9, challenged orally 6 days later with 170 third stage larvae. Group 2 were immunised with two intravenous injections of 200 larvae separated by an interval of fifteen days. These animals were also challenged orally with 170 larvae on day 15 after the last immunisation, whilst mice in group 3 received three injections of 200 larvae intravenously separated by the same time interval as above and challenged orally 15 days after the last injection with a similar number of larvae as group 2. Mice were killed on day 6 and 10 to determine the number of cysts and adult worms.

The results given in Table 4.5 show that mice, after immunisation with larvae as above, were resistant to reinfection. The degree of resistance was related to the number of immunising doses given. It is clear also that treatment of the control groups with carbon tetrachloride had no effect on their susceptibility to infection with this parasite.

In view of the results of the above experiment Balb/C mice and F1 hybrids (C57BL x Balb/C) were immunised intravenously three times

TABLE 4.5 Cysts and adult worms recovered from mice which had been immunised intravenously with 200 third stage larvae and challenged orally with 170 third stage larvae of *N. dubius*.

Numbers of Immunising doses	Cyst numbers ± s.d.*		P**	Adult worm numbers ± s.d.*		P**
	Immune Group	Control Group		Immune Group	Control Group	
1	85±30 (50.0)	151±9	= <0.0005	104±15 (61.1)	164±22	= <0.0005
2	20±14 (11.8)	154±14	= <0.0005	75±18 (44.1)	123±23	= <0.0005
3	4±4 (2.4)	134±15	= <0.0005	28±25 (16.5)	140±23	= <0.0005

* 8 animals were used

** P value from "t" test

(-) percent *N. dubius* recovered

TABLE 4.6 Cysts and adult worms recovered from Balb/C and Swiss White mice which had been immunised intravenously with 3 doses of 200 third stage larvae and challenged orally with 160 third stage larvae of *N. dubius*.

	Cyst numbers*				Adult worm numbers*			
	Balb/C		Swiss		Balb/C		Swiss	
	Immune(1) Group	Control(2) Group	Immune(3) Group	Control(4) Group	Immune(5) Group	Control(6) Group	Immune(7) Group	Control(8) Group
Mean	67.2	160.0	23.0	150.0	80.8	140.2	47.8	133.4
Range	30-101	138-183	6-45	141-161	57-106	116-174	41-59	121-142
S.D.	26.2	15.8	16.0	8.8	19.8	16.1	8.2	8.1
Percentage Recovery	54.5	100.0	14.0	93.95	50.5	87.6	29.9	83.4

P values from "t" test

Group 1 vs group 3 $P < 0.03$

Group 5 vs group 7 $P < 0.01$

* 5-9 mice were used

TABLE 4.7 Adult worms recovered from F_1 mice which had been immunised intravenously with 3 doses of 300 third stage larvae and challenged orally with 160 third stage larvae.

	Adult worm numbers	
	Group 1 Immunised mice	Group 2 Normal mice
Mean	30.75	135.0
Range	1-69	101-160
S.D.	22.7	21.0
Percentage Recovery	19.2	84.4

with 200 larvae as above and their immunity compared with the Swiss White strain. All mice were challenged orally with 160 larvae, fifteen days after the last immunising dose. The results given in Table 4.6 show that Balb/C mice do not respond as well to the immunising schedule as do the Swiss White strain. Table 4.7 indicates that the F1 hybrids developed a resistance comparable to that observed in the latter strain (see Swiss White mice Table 4.6), based on the recovery of adult worms.

6. INDUCTION OF IMMUNITY WITH KILLED THIRD STAGE LARVAE

In general killed larvae of various species of helminths are ineffective vaccines. In order to determine if the same were true of *Nematospiroides dubius* the following experiments were conducted. Infective third stage larvae were killed by repeated (3 times) freezing in ethanol-dry ice for 5 min and thawing at 4°C. Female Swiss White mice were immunised intravenously either two or three times with killed larvae equivalent to 200 living third stage larvae. The injections were separated by intervals of 14 days. Mice were challenged orally with 160 infective larvae on day 10 after the last injection. The number of cysts and adult worms recovered on day 6 and day 10 are given in Table 4.8. The results show that killed larvae were ineffective in inducing immunity to this infection.

This experiment was repeated using different routes of immunisation. Swiss White mice divided into three groups were immunised with three doses of frozen and thawed larvae, each dose being equivalent

TABLE 4.8 Cysts and adult worms recovered from mice which had been immunised intravenously with 200 dead third stage larvae and challenged with 160 third stage larvae of *N. dubius*.

Immunisation	Cyst numbers*		P Value "t" test		Adult worm numbers*		P Value "t" test	
	Immunised Group	Normal Group			Immunised Group	Normal Group		
2 I.V. injections								
Mean	143.8	148.4	=	>.05	136.0	126.8	=	>.05
Range	129-157	137-180			125-158	112-149		
S.D.	10.6	18.5			13.5	14.1		
3 I.V. injections								
Mean	123.0	115.2	=	>.05	107.2	113.6	=	>.05
Range	106-140	84-148			84-136	104-136		
S.D.	13.3	28.8			20.7	10.9		

* 5-7 mice were used.

to 200 larvae, and the time interval between each injection being 14 days. Group 1 was injected intra-muscularly (LM), Group 2, intra-peritoneally (IP), and Group 3 subcutaneously (SC). A fourth group served as controls. All mice were challenged orally with 150 infective larvae on day 10 after the last injection. The immune response as assessed by the recovery of cysts and adult worms is shown in Table 4.9. Once again killed larvae failed to induce resistance to infection.

7. INDUCTION OF IMMUNITY USING ADULT WORMS

It has been reported that implantation of adult worms of *Nippostrongylus brasiliensis* into the intestines of rats will induce immunity to reinfection after the implanted worms have been established (Ogilvie, 1965). Examination of the data of previous experiments where immunity has been demonstrated, it is clear that once the larva has encysted in the immune animal its chances of survival are high. This would suggest that the immune mechanisms operate against the third stage larvae at the time of penetration of the intestinal wall. In view of this it was of interest to see if implantation of adult worms into the intestine would induce immunity to reinfection.

Adult worms were collected as described in Chapter 2 (Section 5) in normal saline. Twenty mice were divided into two groups. 10 mice were used as controls. A laporotomy was performed on each mouse of the second group to expose the small intestine and 200 adult worms were introduced into the lumen via a 20 gauge needle attached to a 1 ml syringe. Two further implantations were made by the same method at 14

TABLE 4.9 Cysts and adult worms recovered from mice which had been immunised with "killed" third stage larvae by various routes and then challenged with 150 third stage larvae of *N. dubius*.

Group	Cyst numbers*				Adult worm numbers*			
	1 I.M.	2 I.P.	3 S.C.	4 -	1 I.M.	2 I.P.	3 S.C.	4 -
Mean	120.8	~~12.0~~ 120	105.8	127.6	99.7	89.4	97.8	106.4
Range	113-134	111-138	94-120	111-150	73-118	62-108	81-119	81-120
S.D.	10.5	10.6	9.4	13.4	17.1	18.6	16.5	15.5

P values from "t" test

All immunised Groups vs controls $P > 0.05$

* 5 mice were used

TABLE 4.10 Cysts and adult worms recovered from mice infected with adult worms and then challenged orally with 120 third stage larvae of *N. dubius*.

	Cyst numbers*		Adult worm numbers*	
	Immune Group	Normal Group	Immune Group	Normal Group
Mean	86.4	95.4	68.0	68.6
Range	63-102	74-120	45-94	61-89
S.D.	16.8	17.4	17.9	10.2

P value "t" test

Immune group vs normal group $P > 0.05$

* 5 mice were used

TABLE 4.11 The percentage of cysts recovered from immunised Swiss White mice challenged orally with 200 third stage larvae of *N. dubius* on day 9, 30, 60, 90, 120, and 150 after the last immunisation.

Challenge (day)	Mean cyst recovery		% Protection
	Immune Group	Control Group	
9	70.8	144.4	50.97
30	53.8	144.4	62.74
60	105.8	197.6	46.48
90	58.4	126.2	53.76
120	49.2	121.2	59.45
150	125.2	145.6	14.05

day intervals. Adult worms from each previous infection were eliminated with carbon tetrachloride 4 days prior to each new implantation. On day 9 after the third implantation the adult worms were eliminated using the above treatment and six days later the mice were challenged orally with 120 infective larvae. The mice of the control group were also operated and treated but no worms were introduced. The recovery of cysts and adult worms from the mice (Table 4.10) shows that the adult worms had not induced resistance to reinfection in the host.

8. DURATION OF IMMUNITY TO *Nematospiroides dubius* INFECTION FOLLOWING IMMUNISATION WITH THIRD STAGE LARVAE

Mice were immunised intravenously three times with 200 infective third stage larvae as previously described. They were then divided into groups of 12 and challenged orally with 200 infective larvae on days 9, 30, 60, 90, 120 and 150 after the last immunising dose. The protection expressed as a percentage was calculated from the

$$\frac{\text{mean count of control group} - \text{mean count of immune group}}{\text{mean count of control group}} \times 100$$

The results given in Table 4.11 show that immunity was still apparent at 120 days but had been lost by 150 days.

9. SPECIFIC-HUMORAL ANTIBODY TITRES IN MICE IMMUNISED WITH THIRD STAGE LARVAE OF *Nematospiroides dubius*

Many species of nematodes stimulate the production of antibodies

though the amounts produced are usually small. However, Panter (1967) failed to detect specific antibodies in the serum from mice immune to N. dubius using a haemagglutination technique involving tanned sheep erythrocytes sensitised with a saline extract of adult worms.

Because part of the present study was designed to measure the importance of specific antibody in immunity to infection by this parasite, it was necessary to establish that following immunisation, antibody could be detected in the serum.

Ten mice were immunised as described in Chapter 2, Section 3 and 10 days after the last immunising dose each mouse was bled from the retro-orbital plexus. The antibody titres were measured using the haemagglutination technique described in Chapter 2, Section 8. The results presented in Table 4.12 show that the one could detect antibody in the serum of these animals although the response between individuals was very variable. Pre-immunisation bleeds were negative.

10. HISTOPHATOGENIC CHANGES IN THE INTESTINE OF IMMUNISED MICE AFTER INFECTION WITH *Nematospiroides dubius*

Mice were divided in three groups of 10. One group was immunised intravenously once with 200 third stage larvae, Group 2 twice with a similar number of larvae, and Group 3, three times. The interval between injections was fourteen days. Fourteen days after the last injection the mice were challenged orally with 200 infective larvae.

TABLE 4.12 Serum antibody titre in individual Swiss White mice immunised with 3 doses of 200 third stage larvae of *N. dubius* intravenously.

Animal number	Antibody titre in mice after 3 doses of immunisation
1	9*
2	27
3	81
4	81
5	243
6	243
7	27
8	9
9	81
10	81
Mean	82.2
S.d. of mean	75.3

* Expressed as the dilution of serum showing complete agglutination of sensitised erythrocytes.

Seven days after challenge half the mice in each group were killed, and the small intestine removed and treated as in Chapter 3, Section 6.

In general the histological changes observed in the area surrounding the cyst were similar to that seen in non-immunised mice though the cellular infiltration and inflammation was more intense. Granulocytic cells were very prominent as were macrophages and monocytes. The inflammation extended a long way from the cyst, though the lamina propia and around the crypts of Lieber Kuhn (Figures 4.1; 4.2. Smears of the cyst content at this stage were carried out as in Chapter 3, Section 6 and results showed an increase in the number of neutrophils, eosinophils, macrophages and monocytes in immune mice (Figure 4.3). Fourteen days after challenge the remaining mice were killed and the small intestins were collected for histological section as described above. Once again the main difference when compared to the situation observed in non-immunised mice was the increase in the number of cells within the empty cysts particularly neutrophils and eosinophils and macrophages. There did not appear to be, at a histological level, any striking relationship between the number of immunising doses and the intensity of the cellular infiltration.

11. CONCLUSIONS

Pre-treating mice either with <u>Salmonella enteritides</u> IIRX or a lipopolysaccharide from a gram-negative strain of bacteria, agents which are known to increase the resistance of mice to bacterial infections,

increased, though only slightly, the resistance of mice to infection with *N. dubius*. This will be commented on later in the Discussion. It has been found that good immunity may be established in these animals following intravenous injection of living larvae. In contrast, killed larvae are ineffective whatever the route of immunisation. Likewise adult worms transplanted into the small intestine of mice are unable to increase the resistance of the host to subsequent re-infection.

Specific humoral antibody may be demonstrated in the serum of mice following immunisation intravenously with living larvae.

FIGURE 4.1

T.S. of the small intestine showing the histopathological changes in an immune mouse 7 days after challenge. Note a marked cellular infiltration of macrophages, lymphocytes and polymorphonuclear cells around the cyst (C) containing the larval stage (L).

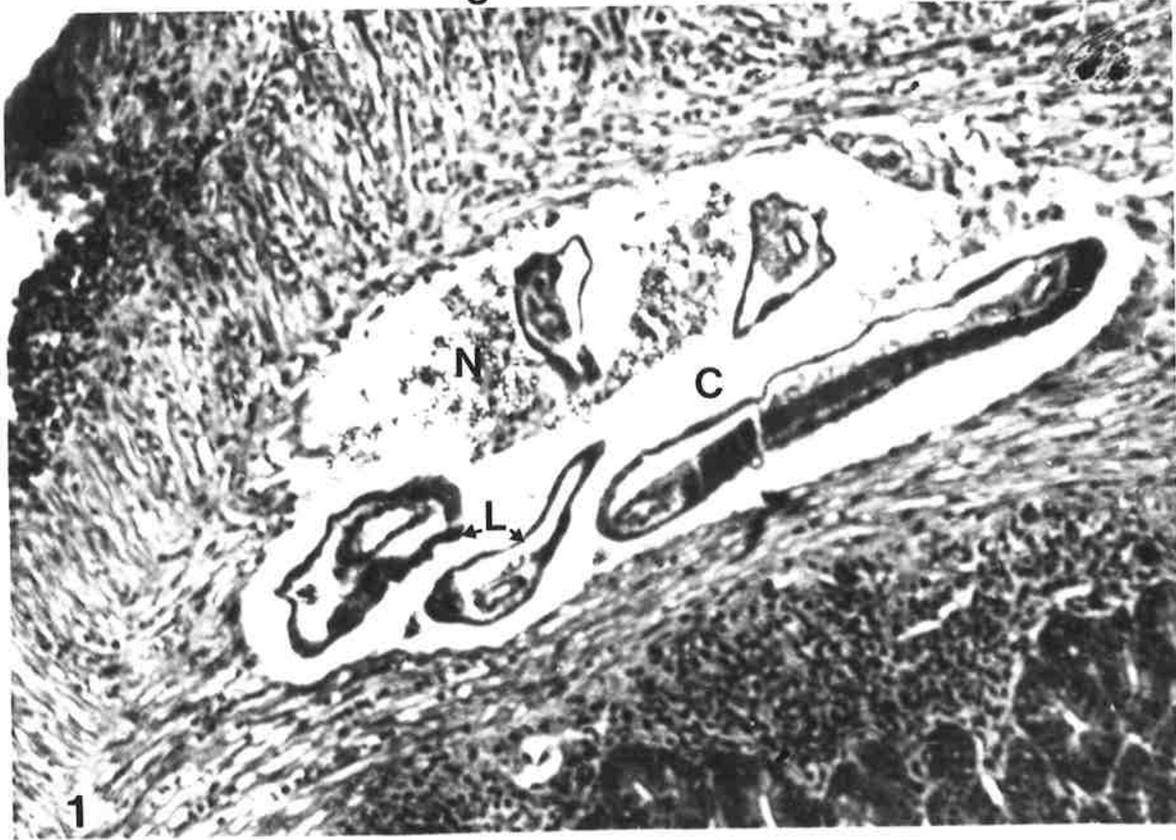
LY = Lymphocyte

M = Macrophage

N = Polymorphonuclear cell

1. Haematoxylin-eosin 170X
2. Haematoxylin-eosin 1100X

Figure 4.1



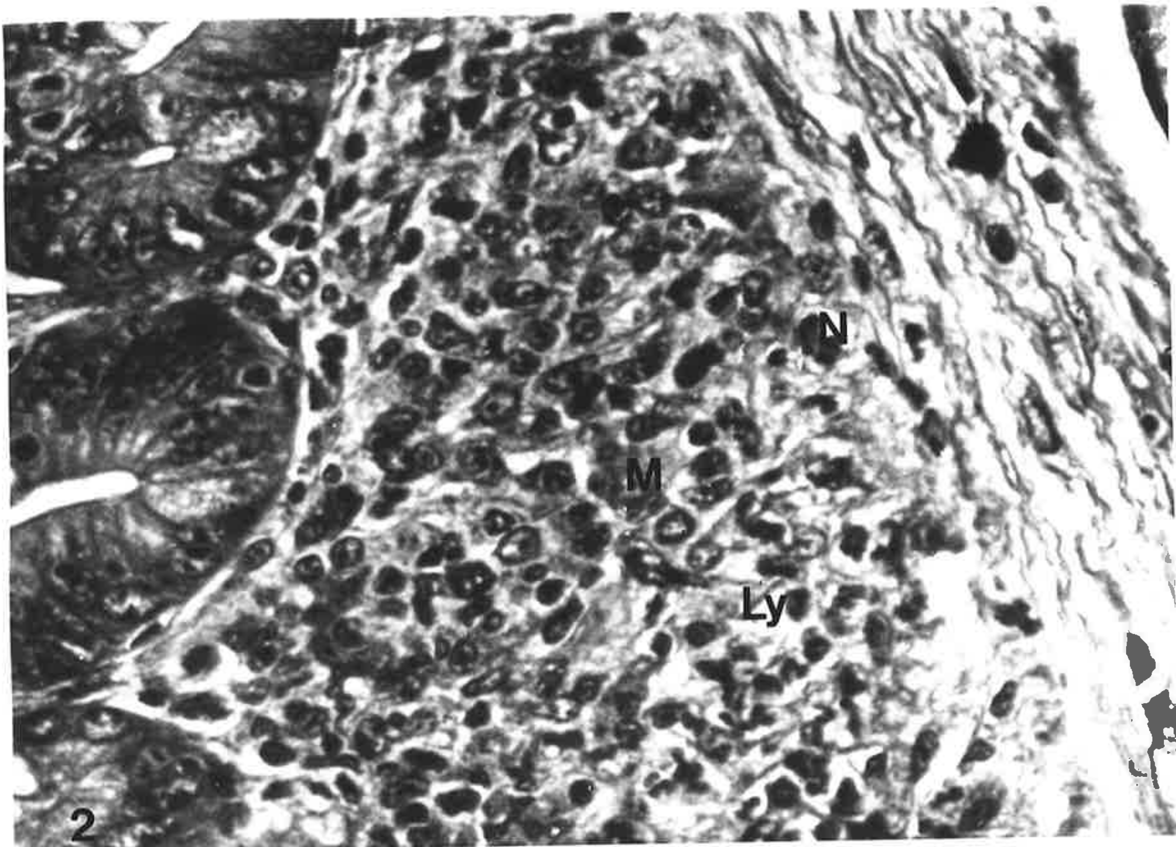


FIGURE 4.2

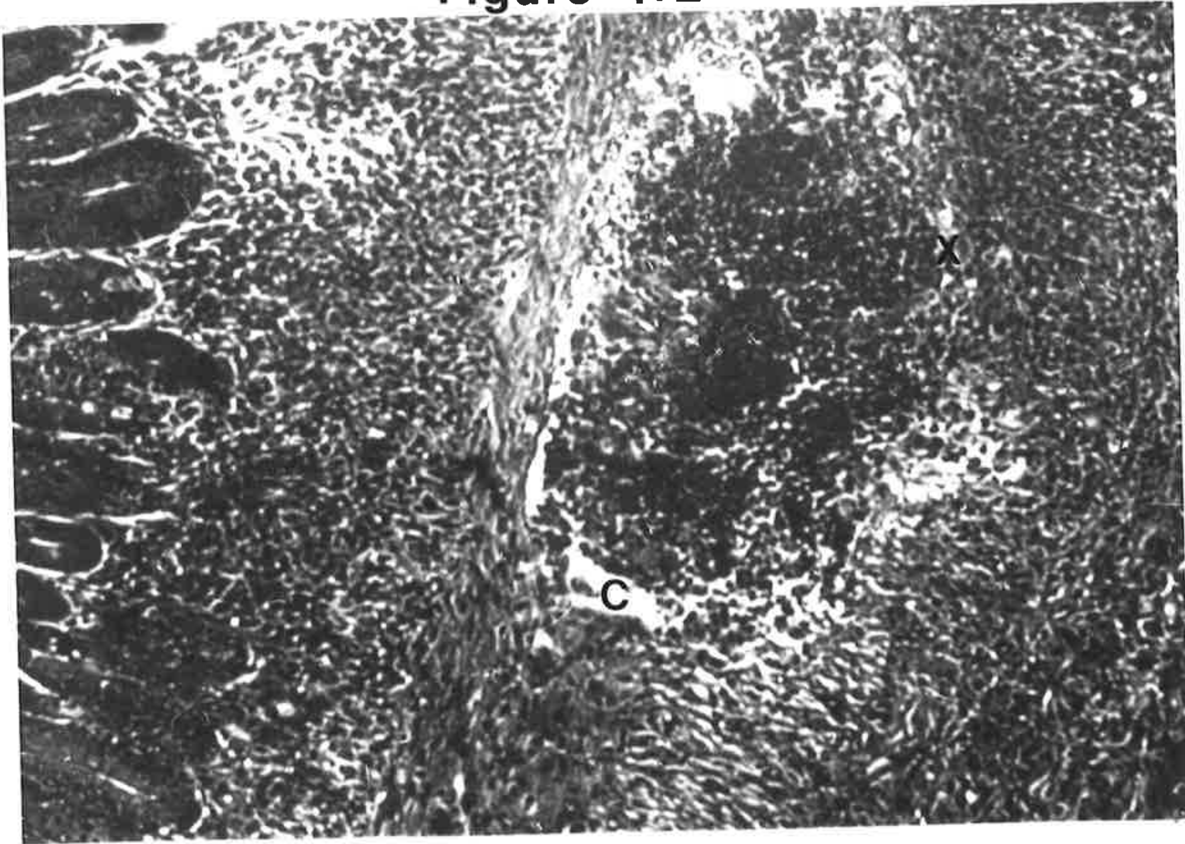
T.S. of the small intestine from an immune mouse showing the histopathological changes 15 days after challenge infection. Note a marked cellular infiltration of macrophages and polymorphonuclear cells mainly inside the cyst (C).

M = Macrophages

N = Polymorphonuclear cells

1. Haematoxylin-eosin 170X
2. Haematoxylin-eosin 1100X

Figure 4.2



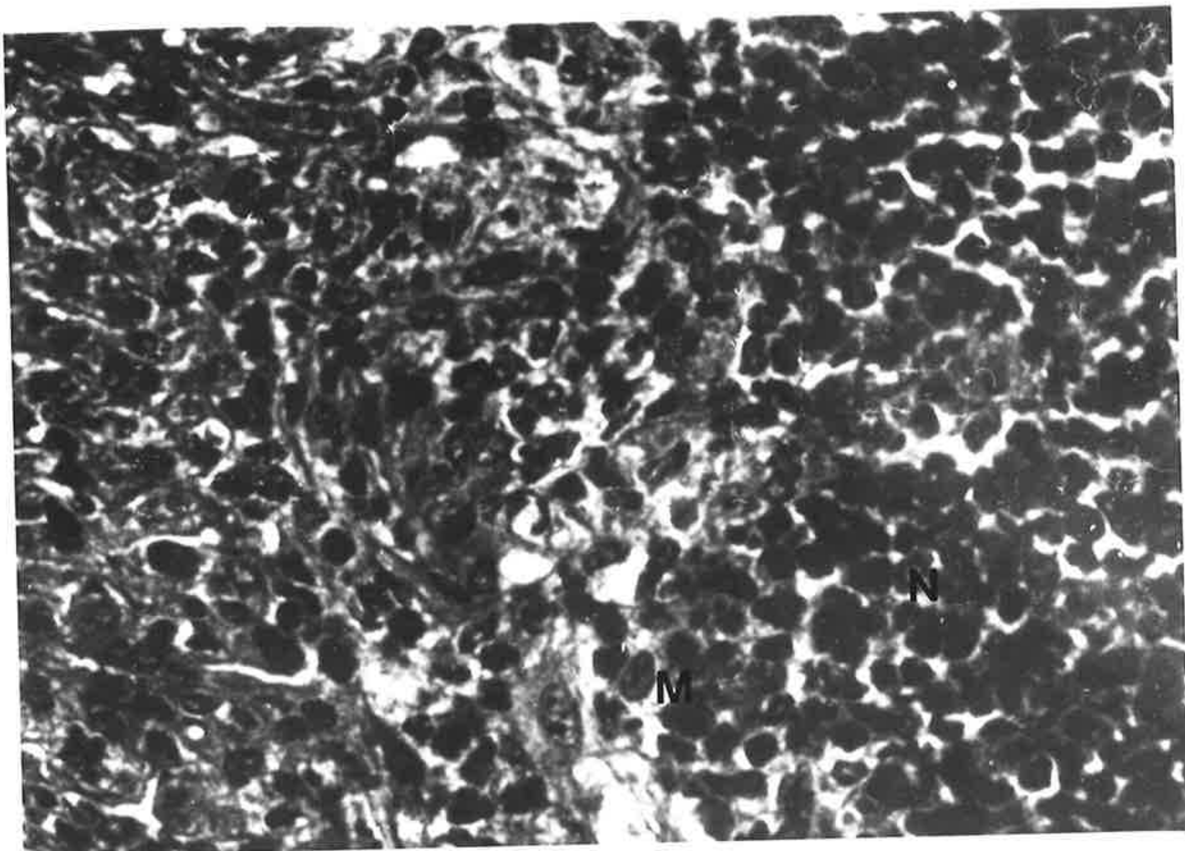


FIGURE 4.3

Cyst smears from the wall of the small intestine of mice 7 days after challenge, showing cell types.

1. Normal mice
2. Immune mice

M = Macrophage

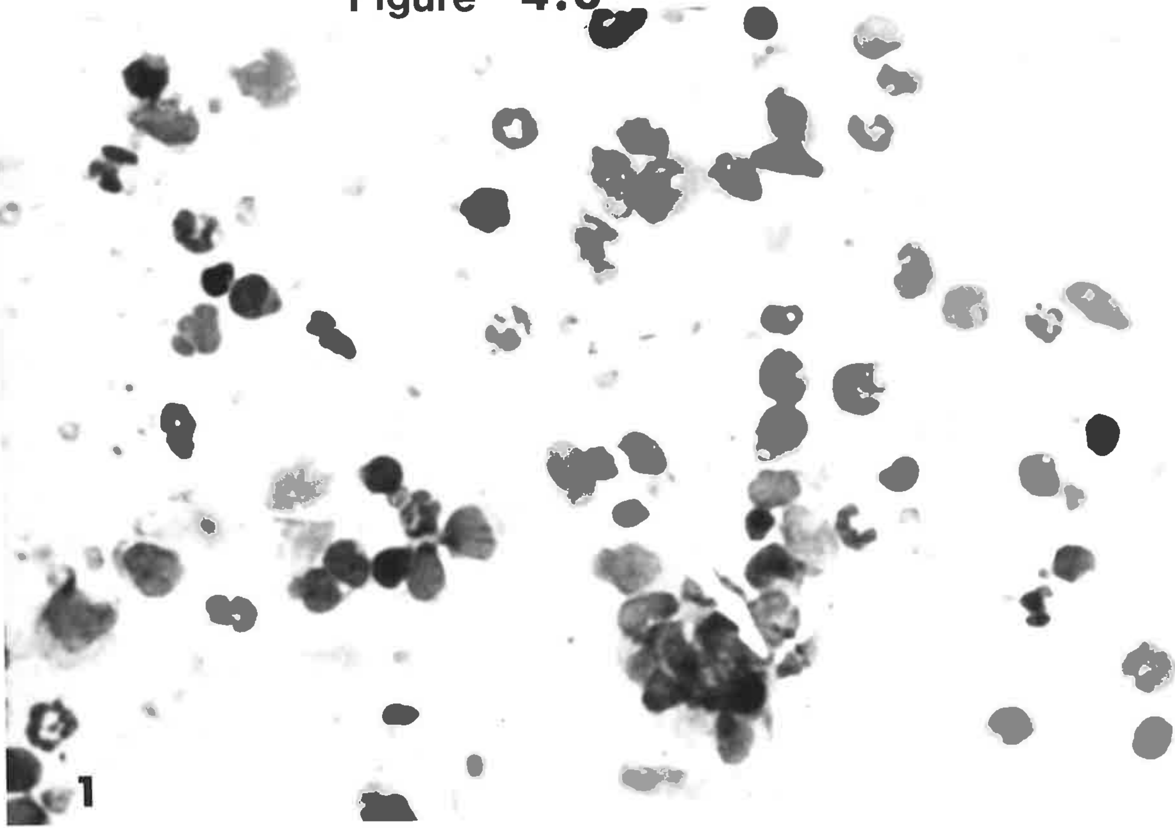
N = Polymorphonuclear cell

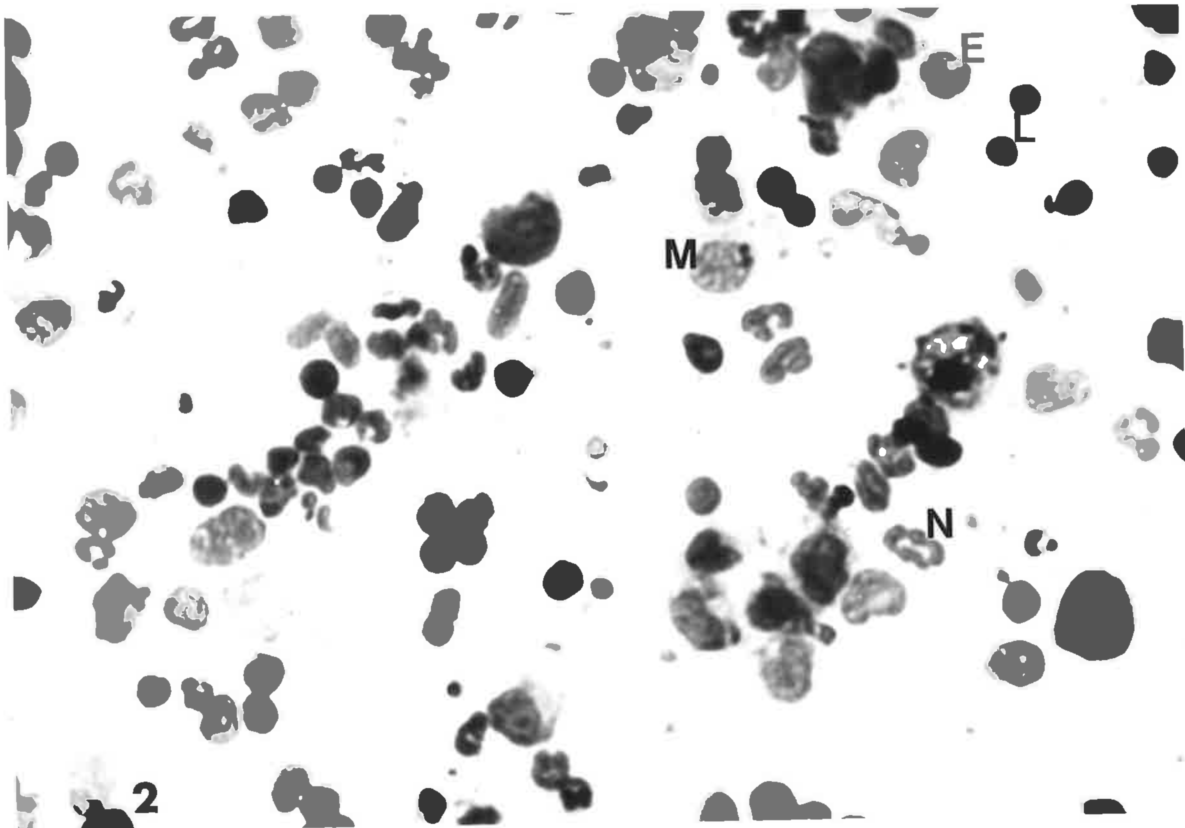
E = Eosinophil

L = Lymphocyte

Wright's stain 440X

Figure 4.3





CHAPTER 5

HUMORAL ASPECTS OF IMMUNITY TO *Nematospiroides dubius*

1. INTRODUCTION

It is well established that nematode infections elicit a specific antibody response in the infected host. The antibody titre may vary tremendously depending on each host-parasite system. The important question is whether these antibodies play a role in host resistance to these particular infections, and if they do, what is the mechanism involved.

Previous work (Chapter 4, Section 5) demonstrated that adult mice may be protected from *N. dubius* infections by active immunisation with infective third stage larvae injected intravenously. Also, it has been reported (Panter, 1967, 1969b; Cypess, 1970) that, transfer of serum from mice immune to this infection, to normal recipients, does not confer resistance on the recipients. In view of the controversy existing in the literature regarding the function of specific antibody in host resistance to nematode infections it seemed pertinent to assess the importance of this factor in *N. dubius* infections in mice.

Two approaches were chosen, the one was obviously to repeat work previously done with adult mice and the second was to see if baby mice born of immune mothers were resistant to this infection. Transfer of immunity passively from mother to the new born has been

demonstrated successfully in *N. brasiliensis*, and *Trichinella spiralis* infections (Jones and Ogilvie, 1967; Greenberg, 1971; Duckett, Denham and Nelson, 1972).

PART 1. PASSIVE TRANSFER OF IMMUNITY TO *Nematospiroides dubius* IN ADULT MICE

2. INTRODUCTION

As mentioned above, Panter (1967, 1969b) and Cypess (1970) were unable to transfer immunity passively. In the present experiments the same model was used but a wider variety of methods were tested and larger amounts of immune serum were transferred to the adult mice.

Mouse anti-*N. dubius* serum was prepared as described in Chapter 2 Section 7 by giving 3 intravenous injections of 200 third stage larvae at 14 day intervals. The antiserum was collected on day 10 after the last injection and stored at -20°C until required. Seven- to nine-week-old Swiss White mice of both sexes were used in these experiments.

Mice were challenged each with 150 third stage larvae orally and divided into 4 groups of 10. Group 1 was given a single dose of specific antiserum on day 1, group 2 on day 2, group 3 on day 4 and group 4 on day 6 after challenge. Physiological saline was used to treat an equal number of control groups in the same manner. In each of the experimental groups the mice received intravenously 0.5 ml of the antiserum. Five days after challenge infection, 5 mice in each group were killed to

determine the number of cysts and the remainder were killed five days later in order to establish the number of adult worms. The results of this experiment are given in Table 5.1 and show that the serum had no effect on the course of the infection.

Because the normal route of infection with this nematode is oral, and as it has been well documented that immunoglobulins readily traverse the intestinal mucosa, the local concentration of specific immunoglobulin in the area perhaps may be important in determining host resistance or susceptibility. In order to test this possibility the following experiments were designed. Mice were divided into 3 groups of 6 or 7. One group was given orally 0.4 ml of specific antiserum 30 min before challenge with 220 third stage larvae/mouse, and at the time of challenge. Nineteen hr after challenge each mouse in this group was fed 0.4 ml of the antiserum and 20 hr later 1.2 ml were given by the same route.

In the case of the other two groups one received normal serum and the other saline. Five days after the infection the mice in each group were killed to determine the number of cysts. The results of this experiment, given in Table 5.2, show that once again the serum from immune mice failed to give any protection. Similar results were obtained when mice received 2.5 ml of antiserum daily for 5 days after infection.

TABLE 5.1 Numbers of cysts and adult worms recovered from mice injected intravenously with serum from immune animals at different time intervals following challenge with infective third stage larvae of *Nematospiroides dubius*.

Experimental and control group number	Treatment of group			
	Specific Antiserum		Saline	
	Number of Cysts	Number of Adult worms	Number of Cysts	Number of Adult worms
1	*119±9 (109-131)	115±15** (99-141)	122±11 (104-132)	116±18 (108-137)
			(108-141)	(107-129)
2	123±7 (109-132)	105±6 (89-122)	123±9 (101-142)	101±7 (83-125)
3	115±5 (102-128)	119±5 (109-137)	117±5 (111-131)	129±9 (111-149)
4	-	112±6 (89-121)	-	124±4 (108-143)

** Standard deviation of mean

(-) = range

* Mean cyst and adult worm recovery

TABLE 5.2 Numbers of cysts recovered from mice dosed orally with serum from immune animals at different times before and after challenge with infective third stage larvae of *N. dubius*.

	Treatment of group		
	Group 1 Specific antiserum	Group 2 Normal serum	Group 3 Saline
	194	197	198
	215	205	239
	207	221	212
	228	204	183
	219	212	260
	195	218	175
	230		192
Mean	212.6	209.5	208.4
S.D. of mean	14.6	9.1	30.9

3. THE EFFECT OF SERUM FROM IMMUNE MICE ON THE INFECTIVITY OF EXSHEATHED THIRD STAGE LARVAE

Some workers have demonstrated the formation of an "antigen-antibody complex" around the orifices of larval and adult nematodes including N. dubius after incubation in a specific antiserum (Panter, 1967). Although Thorson (1954) found that specific antiserum reduced the infectivity of N. brasiliensis larvae, Panter (1967) who used N. dubius obtained negative results. It was thought desirable to repeat the work of Panter. Approximately 150 exsheathed third stage larvae, in 0.2 ml of 199 medium were added to 0.1 ml of either the antiserum, normal serum or 199 medium. The tubes were gassed with 5% CO_2 in air and incubated at $37^{o}C$ in a water bath for 2 hr.

Mice were divided into groups of 10 or 11. In group 1, each mouse received 150 exsheathed third stage larvae that had been incubated in serum from immune mice, group 2, larvae that had been incubated in normal serum and group 3, larvae that had been incubated in 199 medium. Five days after infection 5 mice of each group were killed to determine the number of cysts and another 5 days later the remainder were killed to recover the adult worms. The numbers of cysts and adult worms are shown in Table 5.3. The results indicate that serum from immune mice did not affect the infectivity of exsheathed third stage larvae.

TABLE 5.3 Numbers of cysts and adult worms recovered from mice dosed with exsheathed third stage larvae which had been incubated in either antiserum, normal serum or 199 medium at 37°C for 2 hr.

	Treatment of group					
	Group 1 (Antiserum)		Group 2 (Normal serum)		Group 3 (199 medium)	
	Cysts	Adult worms	Cysts	Adult worms	Cysts	Adult worms
	148	100	137	126	114	109
	120	116	141	104	121	101
	149	73	142	91	134	100
	110	118	137	74	156	63
	150	102	132	123	115	107
		109				106
Mean	135.4	103.0	137.8	103.6	128.0	97.7
S.D. of mean	18.9	16.4	3.9	21.8	17.6	17.3

S.D. = Standard Deviation

4. THE EFFECT OF BY-PASSING THE STOMACH ON THE INFECTIVITY OF EXSHEATHED THIRD STAGE LARVA FOLLOWING INCUBATION IN SERUM FROM IMMUNE MICE

Previous data have failed to show that specific antibody administered passively either intravenously, orally or associated with the larvae afford any protection to the host. One of the possibilities for this failure might be due to the effect of the enzyme pepsin and the gastric acidity on the immunoglobulins. In order to investigate this, the following experiment was carried out.

Approximately 250 exsheathed third stage larvae of *N. dubius* in 0.2 ml of 199 medium were incubated in 0.2 ml of either antiserum, normal serum or 199 medium in individual tubes and treated as described in the last experiment. These treated and exsheathed third stage larvae were then injected directly into the small intestine of normal Swiss White mice of 8 weeks of age under anaesthesia as described in Chapter 2 Section 2.2.

The results given in Table 5.4 indicate that once again serum from immune mice did not affect the infectivity of exsheathed third stage larvae.

5. THE EFFECT OF GUINEA PIG COMPLEMENT ON THE INFECTIVITY OF EXSHEATHED THIRD STAGE LARVAE AFTER INCUBATION WITH SERUM FROM IMMUNE MICE

Previous experiments (Chapter 4, Section 5) have indicated that

TABLE 5.4 The effect of serum from immune mice on the infectivity of exsheathed third stage larvae of *N. dubius* delivered directly into the small intestine of normal mice, following incubation in antiserum, normal serum and 199 medium.

	Treatment of exsheathed third stage larvae		
	Group 1 (Specific antiserum) Nos. of cysts	Group 2 (Normal serum) Nos. of cysts	Group 3 (199 medium) Nos. of cysts
	168	182	234
	201	198	192
	244	242	168
	207	167	234
	205	185	152
		218	179
Mean	205.0	198.7	193.2
S.d. of mean	26.9	27.3	34.3

P value "t" test

among groups $P > 0.05$

the immune mechanism appears to operate on the larval stage possibly at the time it penetrates the intestinal wall. One possible mechanism to consider is the effect that complement may have on larvae that have been in contact with specific antibody. It is known that mouse serum is low in haemolytic complement due to the presence of an inhibitor, so in order to maximise the effect of this system, if such existed, guinea pig serum was used as the source of complement.

Approximately 170 exsheathed third stage larvae in 0.2 ml of 199 medium were added to individual tubes containing either 0.1 ml of serum from immune mice, 0.1 ml of serum from immune mice plus 0.1 ml of a 1/2 dilution of guinea pig serum or 0.1 of 199 medium. They were incubated as described above.

Mice were divided into 3 groups of 5, group 1 was given orally exsheathed third stage larvae which had been incubated in immune serum plus guinea pig complement. Groups 2 and 3 were given orally exsheathed third stage larvae which had been incubated in immune serum and 199 medium respectively. They were killed on day 5 after the infection to determine the cyst numbers as shown in Table 5.5.

The results show that there was some reduction in the number of cysts in mice receiving exsheathed third stage larvae that had been treated with immune serum and guinea pig complement *in vitro*.

6. THE EFFECT OF ANTI-COMPLEMENTARY AGENT "ZYMOSAN"

As it seemed likely that complement was involved in a reduction

TABLE 5.5 The cyst recovery from mice received exsheathed third stage larvae of *N. dubius* which had been incubated in serum from immune mice plus guinea pig complement.

	Treatment of exsheathed third stage larvae		
	Group 1 Specific antiserum plus complement	Group 2 Specific antiserum alone	Group 3 199 medium
	82	155	126
	67	149	118
	132	161	135
	120	125	153
	79	145	156
Mean	96.0	147.0	137.6
S.E. of mean	12.7	4.5	7.4

P value of "t" test

Group 1 vs 3 $P < .025$

Group 1 vs 2 $P < .005$

Group 2 vs 3 $P > .05$

in the infectivity of N. dubius as shown in the last experiment, the following experiment was designed to see whether zymosan (an anti-complementary agent) could reduced the resistance of immune mice to the infection.

Immunised Swiss White mice were treated intraperitoneally with Zymosan (a yeast cell wall fraction, Sigma Chemical Company Ltd.) at a dose rate of 2 mg/mouse according to the following schedule. Group 1 was treated on the same day as the challenge, group 2 on the third day after challenge, group 3 daily from day 0 to day 6 and group 4 serving as immune control mice received normal saline, and group 5 were uninfected mice serving as normal controls. Mice were challenged orally with 200 larvae.

The numbers of cysts and adult worms recovered are shown in Table 5.6. Results indicate that immune mice treated with zymosan were as resistant to infection as the untreated controls.

PART 2. PASSIVE TRANSFER OF IMMUNITY FROM IMMUNE MOTHERS TO THEIR NEONATES

7. INTRODUCTION

Apart from the results using guinea pig serum as the source of complement, all the data indicate that humoral antibody (i.e. antibody circulating freely) plays an unimportant role in protecting the host against infection. However, perhaps a more natural way of assessing its importance before dismissing it as playing only a minor role in

TABLE 5.6 Cysts and adult worms recovered from immunised mice which had been dosed with zymosan before and during the infection. All mice were challenged orally with 200 infective larvae.

	Number of Cysts					Number of Adult worms				
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 1	Group 2	Group 3	Group 4	Group 5
Mean	55.4	51.6	73.0	57.4	160.4	89.6	68.2	85.6	79.0	130.6
Range	42-75	34-76	51-86	31-81	125-175	72-101	51-85	50-103	69-88	121-141
S.D.	14.3	16.4	14.0	19.8	30.1	11.1	15.8	21.1	8.9	7.4

P value "F" test

Groups 1, 2, 3 vs 4 $P > 0.05$

protection is to see if immunity can be transferred from the mother to the neonate, as has been described for certain other nematode infections.

8. THE INFECTIVITY OF *Nematospiroides dubius* LARVAE IN ONE-TO THREE-DAY-OLD BABY MICE

Fifty, two-day-old baby Swiss White mice born to normal mothers were challenged orally with 90 third stage larvae of *N. dubius* in 0.1 ml of distilled water. The oral challenge was given by means of a blunt tip 25 gauge needle attached to a tuberculin syringe. The baby mice were caged with their own mothers and then killed to determine the numbers of cysts and adult worms on days 5 and 10 after challenge.

The results given in Table 5.7 shows that about 45% of the larvae formed cysts the majority of which developed into adult worms. These figures are much lower than those obtained from adult mice (80 to 90% cysts and 68 to 80% adult worms). This may be due to physiological and anatomical differences between the gut surface of baby mice as compared with adult mice (Dobson, 1961). In fact the structure of intestinal epithelia of baby mice may be distinguished from that of adults in that villi of the gut are much shorter.

9. INFECTIVITY OF *Nematospiroides dubius* LARVAE IN BABY MICE BORN TO IMMUNE MOTHERS

Two-day-old baby mice either from immune or normal mothers as described in Chapter 2 Section 3 were challenged orally with a dose

TABLE 5.7 The recovery of cysts and adult worms of *N. dubius* in normal 2-day old baby mice that had received orally 90 third stage larvae.

	Cyst recovery		Adult worm recovery	
	57	14	45	5
	42	32	67	19
	55	23	42	49
	30	45	35	56
	40	8	54	50
	69	47	16	
	38	38	45	
	41	59	60	
	20	17	50	
	41	88	55	
	37	6	17	
	39	7	6	
	77	60	34	
	20	29	5	
	69		59	64
Mean	39.6		39.7	
S.D. of mean	21.2		20.4	
Percentage recovery	45.1		43.0	

of 75 third stage larvae of *N. dubius*. They were left with their own mothers and then killed on either day 5 or 10 to determine the numbers of cysts and adult worms.

The results given in Table 5.8 show that the percentage recovery of cysts from baby mice born to immune mothers was much lower than that obtained from baby mice born to normal mothers. The data given in Table 5.9 show the percentage of adult worms recovered and confirm the data obtained from adult mice in so far as the immunity is expressed against the larval stage and not against the adult worm.

10. TIME OF TRANSFER OF IMMUNITY FROM MOTHER TO YOUNG

It is known that the immunoglobulins of the mouse may be transferred to the young either whilst *in utero* or after birth *via* the colostrum (Brambell, 1970). It was of some interest to determine whether the neonate derived sufficient specific antibody from its mother whilst *in utero* to protect it from this infection or whether it had to be continually supplied after birth. This question was investigated by the following experiment.

Mice were immunised as described in Chapter 2 Section 3. These immunised animals were mated 7 days after the last injection, as were an equal number of untreated female mice. Within 24 hr of birth, the mice born to immune mothers were transferred to normal mothers and their offspring of a similar age transferred to the immune females. Immune and normal mothers with thier own young served as controls.

TABLE 5.8 Numbers of cysts recovered from 2-day-old baby mice born to normal and immune mothers following challenge with 75 third stage larvae orally.

Experiment Number	Mean percent cyst recovery		P value ("t" test)
	Immune group	normal group	
1	*17.80 (5)**	47.50 (8)	P <0.0005
2	9.50 (5)	50.10 (7)	P <0.0005
3	22.06 (6)	58.20 (6)	P <0.0005
4	6.00 (5)	26.66 (5)	P <0.0025
5	5.30 (6)	44.70 (12)	P <0.0005
Average Percentage	12.1	45.4	

** Number of baby mice used

* Mean percent

TABLE 5.9 Numbers of adult worms recovered expressed as a percentage of the challenge dose from 2 day old baby mice born to normal and immune mothers and dosed orally with 75 third stage larvae.

Experiment Number	Mean percentage adult worm recovery		P value ("t" test)
	Immune group	Normal group	
1	*23.10 (8)**	37.90 (6)	P < 0.05
2	8.97 (7)	36.90 (9)	P <0.001
3	2.71 (4)	22.28 (7)	P <0.001
Average Percentage	11.6	32.4	

** Number of baby mice used

* Mean percent

All neonates in this experiment were challenged *per os* with approximately 60 third stage larvae of *N. dubius* 24 hr after transfer to their foster mothers.

Results shown in Tables 5.10 and 5.11 give the figures for the recovery of cysts and adult worms respectively. The figures in the brackets are the numbers of baby mice used in each experiment. Six out of eight experiments in this table show quite clearly that immunity to *N. dubius* may be transferred post-natally. There was no significant difference between the two control groups. It seems therefore that the protection the immune mothers transferred to normal mice was as high as that transferred to their own babies. Once again no immunity could be demonstrated against the adult worms.

One further point to note is that neonates born of immune mothers and suckled within 24 hr on normal mother were not well protected. This suggests that resistance to infection in the newborn to this particular parasite depended on constant intake of immunoglobulins from the milk for a period greater than 24 hr.

11. PASSIVE TRANSFER OF IMMUNITY FROM NORMAL MOTHERS TO NEONATES FOLLOWING INTRAVENOUS INJECTION OF A SPECIFIC ANTISERUM INTO THE MOTHERS

The fact that one could demonstrate successfully passive transfer of immunity from mother to neonate suggested that the previous failure to demonstrate the passive transfer of immunity to adult mice using

TABLE 5.10 Mean cyst recovery, expressed as percentage of the challenge dose, from 2 day old baby mice challenged orally with 75 third stage larvae of *N. dubius*.

Experiment Number	Mean percent cyst recovery			
	Group 1 *	Group 2	Group 3	Group 4
1	-	5.20 (9)	24.30 (7)	-
2	-	5.62 (8)	32.90 (5)	46.71 (6)
3	-	34.70 (7)	37.25 (7)	47.76 (10)
4	-	7.43 (7)	26.16 (7)	19.43 (6)
5	-	14.60 (5)	31.20 (6)	-
6	6.30 (5)	9.16 (7)	63.11 (17)	26.66 (5)
7	5.30 (6)	2.70 (5)	31.00 (5)	44.70 (12)
8	26.32 (5)	46.81 (6)	46.68 (8)	42.88 (5)
Average Percentage	12.6	15.0	36.6	38.3

* 1 immune mother with own offspring
2 immune mother with normal babies
3 normal mother with babies from immune mother
4 normal mother with own offspring

TABLE 5.11 Mean adult worm recovery, expressed as a percentage of the challenge dose, from 2 day old baby mice challenged with 75 third stage larvae of *N. dubius* orally.

Experiment Number	Mean percent adult worm recovery			
	Group 1*	Group 2	Group 3	Group 4
1	0.66 (5)	11.5 (7)	38.16 (7)	80.83 (4)
2	15.30 (10)	43.77 (9)	43.46 (11)	27.92 (8)
3	2.63 (11)	28.31 (9)	54.44 (8)	52.42 (6)
4	1.01 (7)	28.23 (8)	45.24 (9)	50.33 (6)
5	4.99 (10)	1.16 (9)	58.45 (9)	66.26 (6)
6	0.64 (15)	5.96 (8)	65.47 (8)	-
7	1.10 (4)	37.43 (10)	96.94 (6)	-
8				
Average Percentage	3.80	29.6	57.3	46.3

* 1 immune mothers with own offspring
2 immune mothers with babies from normal mothers
3 normal mothers with babies from immune mothers
4 normal mothers with own offspring

serum might be due to a class of immunoglobulin found in the milk which was either not present or in low titre in the serum.

In order to test this possibility pregnant normal Swiss White mice were divided into groups of 3 to 4. Four days prior to parturition group 1 received intravenously daily 0.4 ml of serum collected from immune mice. This regimen was continued for 3 days following parturition. Using a similar dose schedule group 2 received normal mouse serum whilst group 3 received physiological saline. Each baby mouse was challenged orally with 45 third stage larvae of N. dubius 24 hr after birth. The number of cysts and adult worms were ascertained on the fifth and tenth day respectively after challenge.

The results of this experiment, given in Table 5.12, demonstrate quite clearly that the young born of normal mothers that have received serum from immune mice are protected against infection. This is an unusual finding since adult mice themselves are not protected by such treatment.

12. PASSIVE TRANSFER OF IMMUNITY TO *Nematospiroides dubius* BY ANTISERUM IN PREGNANT MICE

The results showing that immunity to this infection may be transferred from normal mothers to their offspring following the passive transfer of serum from immune mice to the nursing female is somewhat surprising in view of the previous results obtained using adult mice. It seemed possible that in addition to antibody the neonatal mice

TABLE 5.12 The effects of mouse anti-*N. dubius* serum on the infectivity of the third stage larvae in 2 day old mice born to normal mothers which had received antiserum passively during pregnancy and after parturition.

Experiment Number	Mean percent cysts recovered			Mean percent adult worms recovered		
	Mothers received			Mothers received		
	Specific Antiserum	Normal Serum	Saline	Specific Antiserum	Normal Serum	Saline
1	7.00	-	23.27	4.00	-	17.00
2	4.90	25.00	30.00	1.70	18.00	21.40

P value, cyst recovery. "t" test	Expt. 1	antiserum vs saline	$P < 0.001$
	Expt. 2	antiserum vs normal serum or saline	$P < 0.001$
P value, adult worm recovery "t" test	Expt. 1	antiserum vs saline	$P < 0.001$
	Expt. 2	antiserum vs normal serum or saline	$P < 0.001$

were receiving other factors from the lactating mothers, the resistance being then a synergistic effect between these and the antibody.

If this were so one might expect lactating females to be more resistant to this infection, providing they were given serum from immune mice, than normal non-lactating females treated in the same manner.

A large batch of female mice were mated and when obviously pregnant divided into two further groups. Twenty four hr following parturition the mice were challenged orally with 220 third stage larvae. At the time of challenge mice in group 1 were given 0.5 ml of specific antiserum intravenously whilst those in group 2 received 0.5 ml of normal serum. Twenty four hr later the mice received intravenously a similar dose of serum. Five days following challenge the mice were killed and the number of cysts recovered compared with those recovered in the control group that had been injected previously as above with normal serum. The data given in Table 5.13 show that lactating females given specific antiserum were as susceptible to the infection as the control groups.

13. CONCLUSIONS

All attempts to transfer immunity passively to adult mice with specific antiserum failed. Similarly infective third stage larvae that had been pre-treated with specific antiserum were still able to undergo development in the host. This would suggest that specific antibody on its own or in collaboration with cells from the normal host does not

constitute an important effector mechanism in determining host resistance to infection.

Infective larvae that had been incubated in the presence of serum from immune animals and guinea pig complement showed a significant loss in infectivity. The possible role of complement in resistance to this infection will be discussed later. In contrast to the above, immunity could be passively transferred from immune mothers to their offspring. The transfer of immunity occurs after birth during the suckling periods. The significance of these observations will be discussed later in the final chapter.

TABLE 5.13 Cyst recovery from lactating mice dosed with antiserum and normal serum at the same time and 24 hours after challenge with 220 third stage larvae of *N. dubius*.

	Treatment of group		
	Group 1 antiserum	Group 2 normal serum	Group 3 non-lactating mice
	Numbers of Cysts		
	178	198	211
	191	208	141
	136	169	161
	186	177	183
	204	197	206
	135	196	159
	169	151	211
	161	155	170
	180	186	207
	169	169	145
	186	131	165
	189		207
			174
			161
Mean	173.7	176.1	178.6
S.d. of mean	21.2	23.7	25.3

CHAPTER 6

CELLULAR ASPECTS OF IMMUNITY TO *Nematospiroides dubius*

PART 1. CELLULAR PASSIVE TRANSFER OF IMMUNITY TO *Nematospiroides dubius*

1. INTRODUCTION

The previous data indicated that in adult mice specific antibody passively administered, neither alone, nor in co-operation with cells of the recipient usually involved in host defence mechanisms, leads to a state of increased resistance against this infection. This situation is not without precedent. It is well documented that immunity against foreign tissues and tumours whilst not independent necessarily of the production of specific antibody requires the involvement of specifically "activated" cells. These cells are usually lymphocytes or macrophages (Evans and Alexander, 1972). The immune mechanisms which act directly against such multi-cellular foreign tissues may play an important role also in immunity to metazoan parasites. Some indication of the direct involvement of cells in this immunity has already been presented in the review (Section 3.3). In order to examine this more thoroughly the following experiments were performed.

2. PASSIVE TRANSFER OF IMMUNITY TO *Nematospiroides dubius* BY SPLEEN CELLS FROM IMMUNE MICE

Seven-week-old male F1 mice (C57BL x Balb/C) were divided into 5 groups of 5 before being treated according to the following schedule.

Each animal in Groups 1, 2 and 3 received intraperitoneally 1×10^8 spleen cells from F1 mice that had been immunised as previously described, group 4 received 1×10^8 spleen cells from normal mice and group 5 received 0.5 ml normal mouse serum by the same route. All mice were challenged with 120 third stage larvae orally, group 1 on day 4, groups 2, 4, 5 on day 7 and group 3 on day 10 following the above treatment. Prior to transfer the cells were washed twice with 199 medium.

According to previous data, immunity to _N. dubius_ in this system seems to be expressed against the larval stage, therefore all mice were killed on day 5 after challenge to determine the cyst recovery. The results shown in Table 6.1 indicate that immunity to _N. dubius_ can be transferred by spleen cells from immune mice.

In view of the above experiment it was of interest to see if immunity could be expressed at a time earlier than that observed previously, namely 4 days. F1 hybrid (C57BL x Balb/C) mice were divided into three groups. Mice in group 1 received intraperitoneally 2×10^8 spleen cells from immunised F1 hybrid mice; those in group 2 received the equivalent of 2×10^8 cells from immune mice that had been ultra-sonicated in ice cold 199 medium for 5 min. Group 3 were injected with 2×10^8 spleen cells from normal mice. All mice were challenged orally with 200 third stage larvae 24 hours after the above treatment. Data given in Table 6.2 on the number of cysts recovered six days after

TABLE 6.1 The numbers of cysts recovered from mice which had received spleen cells from normal and immune mice and challenged orally with 120 third stage larvae of *N. dubius* on day 4, 7 and 10 after the cells were transferred.

	SOURCE OF CELLS				CONTROL
	Immune Mice			Normal Mice	Normal Serum
	Challenge Day				
	4	7	10	7	7
	Number of Cysts				
	82	84	93	106	97
	92	66	79	119	129
	86	55	85	115	129
	112	87	96	138	116
	72	85	69	97	120
Mean	88.8	75.4	84.4	115.0	118.2
s.d. of mean	14.9	14.2	10.9	15.4	13.1

P value "U" test

4 days vs cells from normal mice	P = 0.025
4 days vs normal mouse serum	P = 0.01
7 days vs cells from normal mice	P = 0.005
7 days vs normal mouse serum	P = 0.005
10 days vs normal mouse serum or cells from normal mice	P = 0.005

TABLE 6.2 The numbers of cysts recovered from mice receiving 2×10^8 spleen cells from normal and immune F1 (C57BL x Balb/C) Hybrid mice intravenously and challenged orally with 200 third stage larvae of *N. dubius* 24 hr following cell transfer.

	Treatment of Group		
	Group 1 Cells from immune mice	Group 2 Ultrasonicated cells from immune mice	Group 3 Cells from normal mice
	Cyst Numbers		
	121	171	177
	128	206	198
	173	192	212
	182	184	211
	120	237	195
	116	197	161
	139	-	-
Mean	139.9	197.8	192.3
s.d. of mean	26.9	22.6	20.0

P value "U" test

1 vs 2 P =< 0.005

1 vs 3 P =< 0.005

2 vs 3 P => 0.05

challenge indicate that immunity may be expressed as early as 24 hr but only by living cells. This latter fact rules out the possibility that antibody transferred with the spleen cells collaborates with the recipient's cells in the expression of immunity and suggest a direct involvement between the transferred cells and the larvae.

Since immunity could be transferred with spleen cells taken from mice immunised intravenously with third stage larvae it was of interest to ascertain whether better immunity could be expressed if these cells were able to collaborate with specific antibody against the parasite.

In view of a shortage of F1 hybrid (C57BL x Balb/C) mice an inbred line of CBA mice were used in these experiments. Mice were immunised as previously described, and seven days after the last injection, bled, and their spleens collected. Normal unimmunised mice were treated in a similar fashion.

Male CBA mice of 7 weeks of age were divided into groups and treated in the following manner. Mice in group 1 were injected intravenously with 2×10^8 spleen cells from immune mice, group 2 with a mixture of 2×10^8 spleen cells and 0.2 ml of serum from immune mice, group 3 received the equivalent of 2×10^8 cells that had been ultra-sonicated as above, group 4, 2×10^8 normal spleen cells and group 5, 0.4 mls of saline. All mice were challenged orally with 200 third stage larvae seven days after cell transfer. On day 6 after challenge the mice were killed and the number of cysts determined. Data given

TABLE 6.3 The numbers of cysts recovered from mice which had received spleen cells from immune and normal CBA mice intravenously and challenged orally with third stage larvae of *N. dubius* on day 7.

	Treatment of Group				
	Group 1 cells from immune mice	Group 2 Cells from immune mice and anti-serum	Group 3 Ultrasonicated cells from immune mice	Group 4 Cells from normal mice and normal serum	Group 5 199 medium
	Cyst Number				
	101	117	154	155	203
	88	145	157	165	157
	143	137	158	129	195
	43	143	173	159	162
	49	144	177	168	179
	74	117	174	147	179
	118	101	-	-	-
Mean	88.0	129.1	165.5	153.8	179.2
s.d.	36.1	17.4	10.2	14.3	17.9

P value "U" test

1 vs 2 P = 0.025
1 vs 3 P = 0.001
1 vs 4 P = 0.005
1 vs 5 P = 0.001
2 vs 3 P = 0.001
2 vs 4 P = 0.01
2 vs 5 P = 0.001

in Table 6.3 once again show that mice receiving spleen cells from immune mice are protected against infection with this parasite, but the immunity expressed is not enhanced in the presence of specific antibody, indeed there is a significant reduction. This will be commented on later.

3. PASSIVE TRANSFER OF IMMUNITY TO *Nematospiroides dubius* USING MESENTERIC LYMPH NODE CELLS FROM IMMUNE MICE

An experiment similar to the one above was carried out using mesenteric lymph node cells from immune F1 hybrid (C57BL x Balb/C) mice. The lymph nodes were collected eight days after the last immunising dose. The cells were prepared as described in Chapter 2 (section 6.2) and adjusted to give a concentration of 2.5×10^8 cells/ml. Mesenteric lymph nodes were obtained also from unimmunised mice and treated in a similar fashion. Sixty normal F1 hybrid mice were divided into 6 groups, each mouse in group 1 and group 2 received intravenously 1×10^8 cells from the immune mice, groups 3 and 4 were injected with an equivalent number of cells that had been ultrasonicated for 5 min at 4°C, whilst groups 5 and 6 received 1×10^8 cells taken from normal mice. Groups 1, 3 and 5 were challenged with 220 third stage larvae 24 hr after the above treatment. Whilst groups 2, 4 and 6 were challenged with a similar number of larvae on day 7 following cell transfer.

The data given in Table 6.4 show that immunity may not be transferre

TABLE 6.4 The numbers of cysts recovered from F1 (C57BL x Balb/C) hybrid mice receiving intravenously mesenteric lymph node cells from immune and normal donors and challenged with 220 third stage larvae orally on day 1 and 7 after transfer of the cells.

	Group 1 Cells from immune mice	Group 2 Cells from immune mice	Group 3 Ultrasonicated cells from immune mice	Group 4 Ultrasonicated cells from immune mice	Group 5 Cells from normal mice	Group 6 Cells from normal mice
Challenge Time						
	24 hr	7 day	24 hr	7 day	24 hr	7 day
Cyst Numbers						
	110	141	171	169	231	195
	137	98	177	161	224	181
	150	125	199	174	221	177
	147	131	174	169	224	191
	168	142	152	158	215	204
	136	96		161	208	191
	161	119	147	159	233	205
	129	140	159	171	209	191
	143	143	219		215	
		129	171			
Mean	142.3	126.4	174.3	165.3	220.0	191.9
s.d.	17.3	17.4	22.7	6.2	8.9	9.8

P value	"U" test
1 vs 5	$P < 0.001$
2 vs 6	$P < 0.001$

by ultransonicated cells which suggests that the resistance expressed at 24 hr is unlikely to be due to antibody producing cells in the cell suspension. These results will be discussed further later in the thesis.

The spleen and lymph nodes contain a mixed population of cells but those which might play a direct role in attacking the parasite are sensitised T lymphocytes and/or macrophages. In order to attempt to define the cell type involved, the following experiments were carried out using thoracic duct cells known to be rich in T lymphocytes and peritoneal exudate cells, the predominating cell being the macrophage.

4. PASSIVE TRANSFER OF IMMUNITY TO *Nematospiroides dubius* USING PERITONEAL EXUDATE CELLS FROM IMMUNE MICE

Fl hybrid (C57BL x Balb/C) mice were immunised as before and peritoneal exudate cells were collected from these animals seven days after the last injection. The cells were washed twice in 199 medium and finally injected intraperitoneally into 3 groups of mice so that each mouse received 4×10^6 cells. Three further groups of mice serving as controls were injected by the same route with 0.5 ml of 199 medium. On days 2, 7 and 15 after the transfer of cells, one group and its control were challenged orally with 120 third stage larvae and killed five days after challenge in order to determine the number of cysts.

TABLE 6.5 The numbers of cysts recovered from mice which received peritoneal exudate cells from immune and normal F1 (C57BL x Balb/C) mice intraperitoneally (IP) and challenged orally with 120 third stage larvae of *N. dubius* on day 2, 7 and 15 following cell transfer.

	Time of Challenge/Days					
	2		7		15	
	Group 1 Cells from immune mice I/P	Group 2 199 medium I/P	Group 3 Cells from immune mice I/P	Group 4 199 medium I/P	Group 5 Cells from immune mice I/P	Group 6 199 medium I/P
	Numbers of Cyst Recovered					
	80	77	91	74	86	94
	79	107	74	96	108	119
	80	115	95	104	127	94
	46	72	107	100	94	116
	67	91	86	106	110	101
	75	97	109	86	83	93
	67	80	84			
		96				
Mean	70.6	91.9	92.3	94.3	101.3	102.8
S.d. of mean	12.2	14.9	12.6	12.2	16.8	11.8

P value of "U" test

1 vs 2 $P < 0.03$
3 vs 4 $P > 0.05$
5 vs 6 $P > 0.05$

Data given in Table 6.5 indicate that peritoneal cells were ineffective in transferring immunity.

5. PASSIVE TRANSFER OF IMMUNITY USING THORACIC DUCT LYMPHOCYTES FROM IMMUNE MICE

CBA mice were immunised with third stage larvae as previously described. Seven days after the last injection of larvae the mice were subjected to thoracic duct cannulation and the lymph collected over a period of 24 hr as described in Chapter 2 (section 6.3). After washing in ice-cold 199 medium, the lymphocytes were resuspended in the same medium to give a concentration of 2×10^7 cells/ml. More than 90% of these cells were judged viable by trypan blue exclusion. The suspension of lymphocytes was divided into two equal portions one portion was ultrasonicated at 4°C for 5 min.

CBA mice aged 7 weeks were divided into three groups. Mice in one group received intravenously 0.25 ml of 199 medium containing 5×10^6 viable lymphocytes, the second received an equivalent number of ultrasonicated cells and the third 0.25 ml of 199 medium only. Seven days after this treatment the mice in all groups were challenged orally each with 200 third stage larvae of *N. dubius*. Mice were killed five days after challenge and the number of cysts counted. The results given in Table 6.6 indicate that immunity to *N. dubius* can be transferred to normal mice by thoracic duct lymphocytes from immune mice.

TABLE 6.6 Numbers of cysts recovered from CBA mice receiving thoracic duct lymphocytes from immune CBA mice intravenously and challenged orally with 200 third stage larvae of *N. dubius* on day 7.

	Treatment of Group		
	Group 1 Cells from immune mice	Group 2 Ultrasonicated cells from immune mice	Group 3 199 medium
	Numbers of Cyst Recovered		
	119	173	198
	132	211	190
	168	210	159
	110	186	210
	98	174	164
	164		183
	146		214
	138		190
Mean	134.4	190.8	188.5
S.d.	24.8	18.7	19.7

P value from "U" test

1 vs 2 $P < 0.001$

1 vs 3 $P < 0.002$

2 vs 3 $P > .05$

PART 2. THE EFFECT OF PERITONEAL EXUDATE CELLS ON THE INFECTIVITY OF *Nematospiroides dubius*

6. INTRODUCTION

Previous studies in Part 1 of this chapter showed that peritoneal exudate cells harvested from immune mice failed to protect recipients against this infection when injected intraperitoneally. One reason for this could have been that the cells either failed to home to the areas where the larvae penetrated the intestinal wall, or arrived there in insufficient numbers to damage the parasite. In view of the importance of these cells in determining, for example, immunity to tumours which may be regarded as large parasites, it was decided to explore their effectiveness more thoroughly by actually injecting larvae into the peritoneal cavity under various conditions.

7. THE *IN VIVO* EFFECTS OF PERITONEAL EXUDATE CELLS FROM IMMUNE AND NORMAL MICE ON THE INFECTIVITY OF EXSHEATHED THIRD STAGE LARVAE

Immune and normal Swiss White mice were injected intraperitoneally with 4000 exsheathed third stage larvae. All mice were killed 4 hr. after the injection and the larvae collected by washing out the peritoneal cavity with 199 medium. After washing twice by centrifugation in 199 medium the larvae were finally resuspended in an 0.2% solution of trypsin in 199 medium and incubated at 37°C for 15 minutes. Such a

concentration of trypsin had been shown previously not to effect the infectivity of larvae. The purpose of the trypsin treatment was to remove the large numbers of cells adhering to the larvae recovered from the immune mice. Larvae collected from normal mice were treated in a similar fashion. After this treatment the larvae were washed twice in 199 medium by centrifugation, counted and 320 of them were given orally to normal mice. Five days after challenge the mice were killed and the number of cysts determined. Data given in Table 6.7 show that infectivity of the larvae was affected significantly after exposure to cells in the peritoneal cavity of immune mice.

Whilst these results indicated that larvae that had remained in the peritoneal cavity of immune mice were damaged it did not indicate clearly that peritoneal cells _per se_ were directly responsible for this damage. It was possible that damage was a result of some other factor produced by immune mice which led finally to cells concentrating around the injured larvae. In order to investigate this system further, use was made of millipore chambers. The chamber measured 3 mm in height and were 10 mm in diameter, and could hold a total volume of 0.4 ml. Membranes of two different pore sizes were used namely 3 μ and 0.45 μ. The 3 μ membranes allowed cells to pass into the chamber whereas the 0.45 μ prevented them but allowed the free passage of macromolecules. The membranes were fixed to the chambers using M.F. cement (Millipore Corporation, Bedford, Massachusetts) and were left to dry at least

TABLE 6.7 Numbers of cyst recovered from mice receiving 320 third stage larvae of *N. dubius* which had been incubated in the peritoneal cavity of normal and immune mice for 4 hr.

	Third stage larvae incubated with cells from immune mice	Third stage larvae incubated with cells from normal mice
	Cyst Numbers	
	185	259
	185	314
	189	289
	179	248
	157	262
		214
Mean	179.0	264.3
S.d.	12.8	34.4

P value from "U" test

$P < 0.005$

6 hours before the larvae were placed into the chamber via a 1 mm hole in the side. The holes were sealed with paraffin wax, then with MF Cement, and the chambers implanted into the peritoneal cavity using the technique for exsheathing third stage larvae given in Chapter 2 (section 2.1).

8. THE FATE OF INFECTIVE LARVAE OF *Nematospiroides dubius* IN MILLIPORE CHAMBERS IMPLANTED INTO THE PERITONEAL CAVITIES OF NORMAL AND IMMUNE MICE

It seemed important to establish at first a suitable time to sample the chamber contents and to test the infectivity of the larvae. In order to do this chambers bearing membranes with a pore size of 3 μ were implanted into normal and immune mice. Each chamber contained 300 infective larvae. At various time intervals chambers were removed, the membranes lifted off and the adherent cells and larvae carefully scraped onto microscope slides. The following is a description of the events which took place over a period of 4 days. Peritoneal cells of both normal and immune mice passed into the chamber and reach a maximum concentration at about 24 hour. It was quite clear that the rate of passage of cells into the chamber was more rapid in those planted in immune animals. Few cells were associated with larvae recovered from normal animals but large numbers of cells completely encapsulated larvae taken from immune mice. At this time the larvae were all still alive as

judged by visible movement. At 48 hours larvae taken from chambers implanted in immune mice were inactive within the cell mass and many had irregular cuticles. In contrast larvae from normal mice were all still alive and in many cases were free of adherent cells. By 96 hr. larvae from immune mice showed severe cuticular damage and those recovered from normal mice were now inactive though showing no signs of damage to the cuticle. This is perhaps not surprising because it would be possible for the normal mice,within 4 days,to mount an immune response.

9. THE INFECTIVITY OF LARVAE RECOVERED FROM CHAMBERS IMPLANTED INTO NORMAL AND IMMUNE MICE

In view of the intense cellular infiltration by 24 hr, this time was chosen as suitable for testing the infectivity of the larvae, especially because visual observation had indicated that all the larvae were alive irrespective of the immune status of the host.

Chambers were inoculated with 550 exsheathed third stage larvae and implanted into normal and immune mice. After 24 hr. in these environments the chambers were recovered, and the larvae washed and treated with trypsin as before. Following this procedure a known number of larvae (360) collected from each group of animals were fed orally to normal mice. These animals were killed five days later and the number of cysts determined. The data given in Table 6.8 indicate

TABLE 6.8 The infectivity of third stage larvae recovered from chambers implanted into normal and immune mice.

	*Infectivity of larvae recovered from chambers implanted into normal mice	*Infectivity of larvae recovered from chambers implanted into immune mice
	Number of Cysts Recovered	
	363	72
	348	85
	264	181
	298	177
	372	153
Mean	329.0	133.6
S.d. of mean	46.2	51.6
% cyst recovery	91.3	37.3

* each mouse received 360 third stage larvae

P value "U" test

P = 0.005

that larvae taken from chambers recovered from immune mice, though still alive at the time of transfer to a fresh host, showed a marked loss in infectivity.

10. THE EFFECT OF SPECIFIC ANTISERUM ON THE ABILITY OF PERITONEAL EXUDATE CELLS FROM NORMAL MICE TO INACTIVATE INFECTIVE LARVAE

Despite the fact that previous experiments failed to show that specific antibody transferred passively to adult mice afforded protection against this infection, it was of interest to see if specific antiserum added to chambers containing infective larva and planted into normal mice allowed peritoneal cells under such conditions to react with the larvae.

Three groups of chambers were set up, group 1 contained 500 larvae plus 0.4 ml of specific antiserum, group 2 and group 3 the same number of larvae but the former contained 0.4 ml of normal mouse serum whilst the latter contained 0.4 ml of 199 medium. The chambers were placed inside the peritoneal cavities of normal mice and recovered after 24 hr. The larvae were removed from the chambers and washed and treated with trypsin as described previously. After this treatment a known number of larvae from each of the experimental groups were fed to normal mice. The results given in Table 6.9 show that peritoneal cells of normal mice had no effect on the larvae even in the presence of specific antibody. These data will be discussed later in relationship

TABLE 6.9 The effect of specific antiserum on the ability of peritoneal exudate cells from normal mice to inactivate third stage larvae.

	Treatment of Group		
	Group 1* Infective larvae incubated with serum from immune mice and cells from normal mice	Group 2* Infective larvae incubated with serum and cells from normal mice	Group 3* Infective larvae incubated with 199 medium
	Number of Cysts Recovered		
	261	191	360
	139	352	380
	217	388	295
	291	283	307
	164	355	337
	365	266	
Mean	239.5	305.7	355.8
S.d. of mean	83.9	73.2	34.9
% cyst recovery	47.9	61.1	71.2

* each mouse received 500 third stage larvae

P value from "U" test

1 vs 2 $P > 0.05$

1 vs 3 $P = 0.05$

2 vs 3 $P > 0.05$

to the need of having a specifically sensitised population of cells for the expression of immunity to this infection. Further experiments were designed to explore the possibility that cytotoxic factors may be released from the peritoneal cells of immune animals.

11. THE FATE OF LARVAE WITHIN CHAMBERS WITH MEMBRANES HAVING A PORE SIZE OF 0.45 μ

Mice immune to infection with *N. dubius* were divided into two groups. One group received chambers with membranes having a pore size of 3.0 μ whilst the others received chambers with a pore size of 0.45 μ. An equal number of normal mice were treated in a similar fashion. Each chamber was inoculated with approximately 140 larvae. Twenty four hours later the mice were killed and the larvae within the chambers treated as previously described. Normal mice were challenged orally with these larvae and the number of cysts counted on day 5. The data given in Table 6.10 show that despite the large accumulation of cells on the outer part of the 0.45 μ membranes of chambers planted into immune mice the larvae within the chamber were apparently unharmed. In contrast, larvae recovered from chambers into which the peritoneal cells of immune mice could penetrate, were damaged as measured by a loss of infectivity.

It seemed possible however that cytotoxic factors may be released after contact of the cells with larvae. In order to test this hypothesis

TABLE 6.10 The fate of larvae within chambers with membrane having a pore size of 0.45 μ.

	Pore size of chambers implanted* into immune mice		Pore size of chambers implanted* into normal mice	
	0.45 μ	3.0 μ	0.45 μ	3.0 μ
	Numbers of Cyst Recovered			
	91	53	47	65
	79	15	56	56
	77	55	71	80
	35	3	90	81
	81	55	43	75
	54	50	76	49
Mean	69.5	35.5	63.8	67.7
S.d.	20.8	23.2	18.2	13.2
% cyst recovery	92.7	51.3	85.1	90.2

* each mouse received 75 third stage larvae

P value "U" test

3.0 μ immune vs 3.0 μ normal $P < 0.025$

0.45 μ immune vs 0.45 μ normal $P > 0.05$

0.45 μ normal vs 3.0 μ immune $P < 0.05$

TABLE 6.11 The infectivity of infective larvae recovered from twin chambers separated by membranes such that some larvae were in contact with cells whilst the others were in contact with possible cytotoxic factors released as the result of cell/parasite interactions.

	Larvae in contact with cells*	Larvae in contact with possible cytotoxic factors*
	Numbers of Cyst Recovered	
	78	215
	55	280
	84	206
	176	241
	74	193
	166	205
	12	224
Mean	92.1	223.4
S.d. of mean	58.9	29.3
% cyst recovery	35.4	85.9

* each mouse received 260 third stage larvae

P value "U" test

$P < 0.001$

twin chambers were made which were separated by a 0.45 μ millipore membrane. One side of the double chamber was closed with a membrane of similar pore size whilst the other was covered with a 3 μ millipore membrane. Each compartment of the twin chamber was inoculated with 300 larvae. Such chambers were planted into the peritoneal cavities of seven immune mice. Twenty four hr later the larvae were recovered from the chambers, and, after the treatment described above, fed orally to normal mice. Each mouse was challenged with 260 larvae. One group of mice received larvae from the compartment into which cells could pass whilst the other received larvae from that into which only macromolecules could diffuse.

The results shown in Table 6.11 suggest that soluble cytotoxic factors were not released into the medium after contact of the peritoneal cells with the infective larvae.

PART 3. CELLULAR CHANGES TAKING PLACE ON THE OMENTUM OF IMMUNE AND NORMAL MICE FOLLOWING INJECTION OF INFECTIVE LARVAE INTRAPERITONEALLY

12. INTRODUCTION

Chandler (1936) showed that when the adult form of *N. brasiliensis* was injected into rats intraperitoneally, the worms were found almost exclusively on the great omentum. Similar recent studies

by Greenberg and Wertheim (1973) using the second and third stage larvae of *N. brasiliensis* showed that the larvae on the omentum of immune rats were surrounded with cells. These cells were mainly macrophages and eosinophils.

The mouse omentum according to Fedorko and Hirsch (1969), consists of an apron of varying size extending from the greater curvature of the stomach. The distal end contains a fold of adipose tissue with some blood vessels. However the greater part of the omentum appears as a transparent double membrane. The cells of the membrane are mainly mesothelial cells, with a few macrophages and lymphocytes. The omentum has been used to follow the cellular events that take place during the induction of an immune response after antigen has been given intraperitoneally. (Ax, Kaboth and Fischer, 1966; Kaboth, Ax and Fischer, 1966; Malchow and Fischer, 1969; Hartman and Fischer, 1973; Kasahara, 1971; Hajdu, Holub and Trebichavsky, 1971; Kasahara, 1973).

In view of the results obtained in Part 2 of this Chapter it seemed desirable to follow the cellular events taking place on the mouse omentum following injection of living infective third stage larvae in an attempt to define the cell types associating with the larvae which leads ultimately to its death.

13. EXPERIMENTAL DESIGN

A large group of female Swiss White mice were immunised with third stage larvae as described in Chapter 2 Section 3. Ten days after

the last immunisation, they and an equal number of normal females were injected intraperitoneally with 3000 third stage larvae. Five mice taken from each group were killed at the following times, 2, 10, 30, 60, 90 min, and 6, 12, 24, 48, 72, 96, 120 hr. The peritoneal cavities were washed out as described in Chapter 2 Section 6.4 with 199 medium. The washouts were centrifuged as previously described and the cells resuspended in a small volume of foetal calf serum. Thin smears were made of the suspended cells on microscope slides which after drying in air were stained with Wright's stain. The identification of the cells adhering to the larvae recovered from the peritoneal cavity was carried out under oil immersion. After washing out the peritoneal cavity, the omentum was carefully removed from each mouse. Two omenta from each group were spread out on microscope slides, air dried and stained as above, whilst a further two were fixed in Bouin's fixative prior to sectioning. The remaining one was examined as a fresh wet mount.

14. EXAMINATION OF LARVAE RECOVERED FROM THE PERITONEAL CAVITY

Within 10 minutes after injection, larvae recovered from the peritoneal cavities of immune mice had large numbers of cells adhering to their cuticles. The predominant cell type was a typical macrophage. A few mononucleocytes and eosinophils were also present (Fig. 6.1.1). After three hours the recovered larvae were surrounded by a mass of cells. The numbers of cells associating with the mass increased steadily up to

6 hr. After this time few larvae could be recovered from the peritoneal cavity but could be found associated with the omentum. At three hr macrophages were still the predominant cell type found in the mass of cells surrounding the larvae, but there were also numbers of lymphocytes, eosinophils and polymorphonuclear cells. In contrast to the above, very few cells and these were macrophages, were found associated with larvae recovered from normal animals.

15. CELLULAR CHANGES ON THE OMENTUM AFTER THE INTRAPERITONEAL INJECTION OF THIRD STAGE LARVAE

Prior to the injection of the third stage larvae, omenta taken from immunised mice were indistinguishable from those removed from normal animals (Figures 6.1.2; 6.1.3). However after the injection of larvae striking cellular changes were observed on the omenta taken from immune animals. Ten minutes after injection larvae were found associated with the omenta from both immune and normal mice. A large number of cells mainly macrophages were associated with the larvae lying on the omenta removed from the immune animals (Figures 6.2.1; 6.2.2). By three hours the larvae on the omenta taken from immune mice were in the centre of a mass of cells. Macrophages appeared to be the predominant cell, with some mononucleocytes, lymphocytes, eosinophils and polymorphonuclear cells. Because of the dense mass of cells, it was not possible to obtain any quantitative data as to the relative numbers of the various cell types.

In contrast to the above, larvae on the omenta taken from normal mice had provoked little cellular change (Figures 6.3.1; 6.3.2.). Sections through the omenta of immune mice, three hr after the injection of larvae, showed an increase in the numbers of mesothelial cells as well as a pronounced cellular infiltrate made up of the above cells. (Figures 6.3.3; 6.3.4). At 72 hr the larvae on the omenta from immune mice had irregular cuticles and were inactive. Those on the omenta of normal mice, in contrast, were active and were not surrounded by many cells.

16. CONCLUSIONS

The data indicate that immunity to this infection may be transferred ~~providing~~ with either spleen cells, mesenteric lymph node cells or thoracic duct lymphocytes from immune mice. Immunity could not be transferred with peritoneal exudate cells. However results obtained from studies measuring the loss of infectivity of larvae recovered after injection into the peritoneal cavity of immune animals would suggest that peritoneal exudate cells appears to require intimate contact between the cell and the larvae.

Observations on the cellular changes taking place on the omenta of immune mice after injection of larvae intraperitoneally, show that the macrophage is the predominant cell type associated with the parasite.

These observations will be discussed more fully later.

FIGURE 6.1

1. Peritoneal exudate cells of immune mouse adhering to the third stage larvae of N. dubius 10 min after they had been injected intraperitoneally.

L = Larva

M = Macrophage

E = Eosinophil

Wright's stain 1600X

2. and 3. Omenta from immune and normal mice prior to the intraperitoneal injection of the third stage larvae. Note theere is no difference between them.

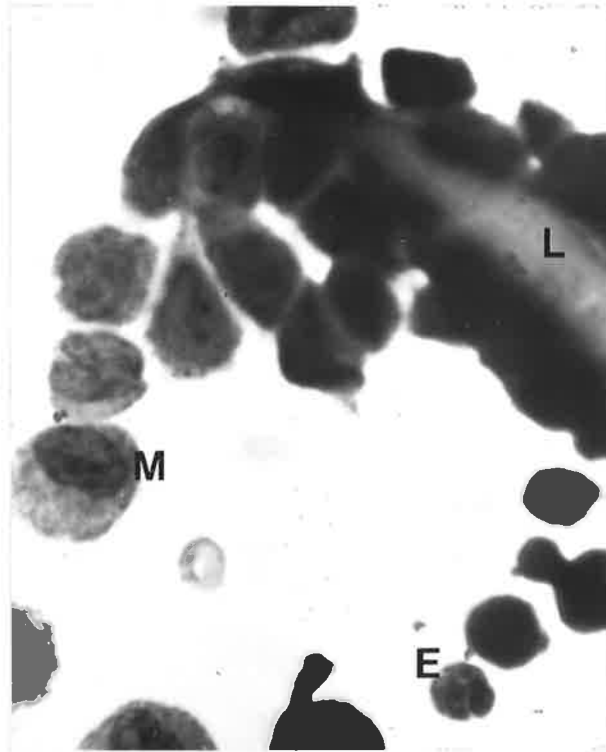
ME = Mesothelial cell

V = Vaccuole

T = Transparent tissue

Wright's stain 100X

Figure 6.1



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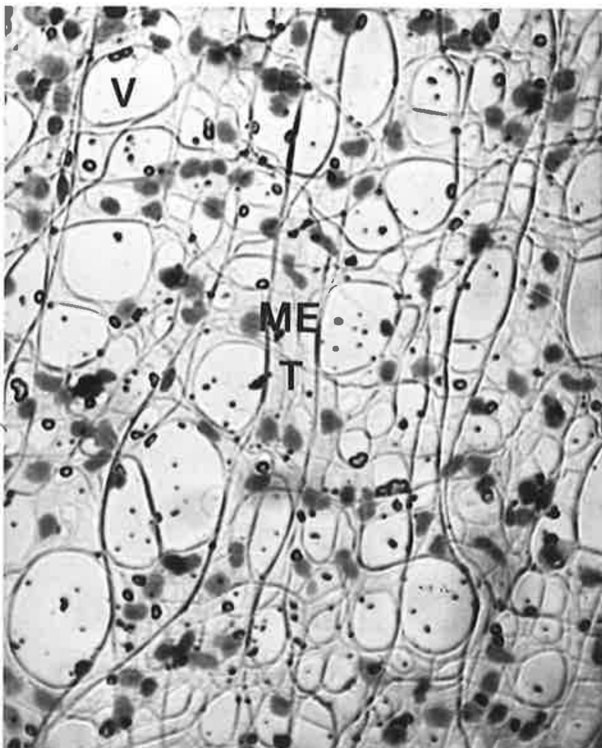

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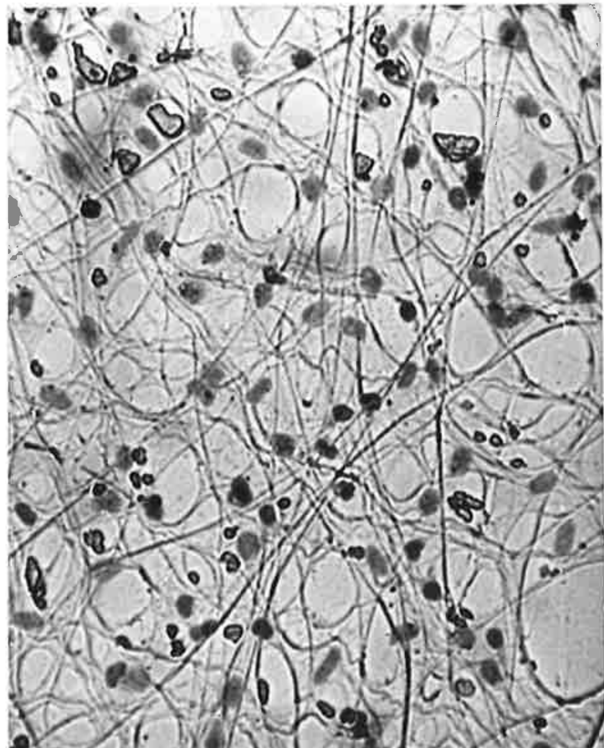


3

FIGURE 6.2

1. and 2. Omenta from immune and normal mice 10 min after the intraperitoneal injection of the third stage larvae. Note the third stage larvae coated with peritoneal cells attached to the omentum of immune mice and the accumulation of cells on this omentum. The normal omentum shows no cellular changes.

L = Larvae

O = Omentum

C = Peritoneal cells

S = Sheath of the third stage larva

Wright's stain 100X

3. and 4. Fresh preparation of menta from immune and normal mice 3 hr after the intraperitoneal injection of the third stage larvae. Note larvae coated with peritoneal cells attached to the omentum of the immune mouse. There were no cells adhering to larvae attached to the omentum of normal mouse.

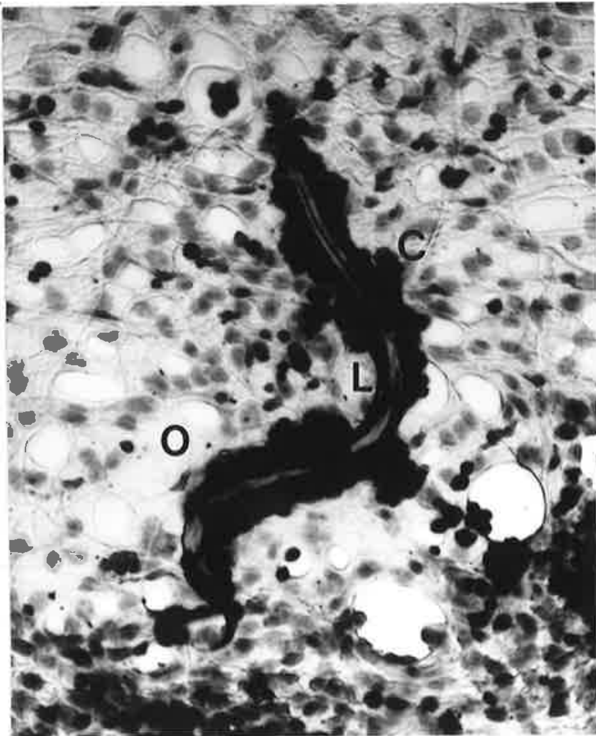
L = Larvae

O = Omentum

C = Peritoneal cells

Wright's stain 100X

Figure 6.2



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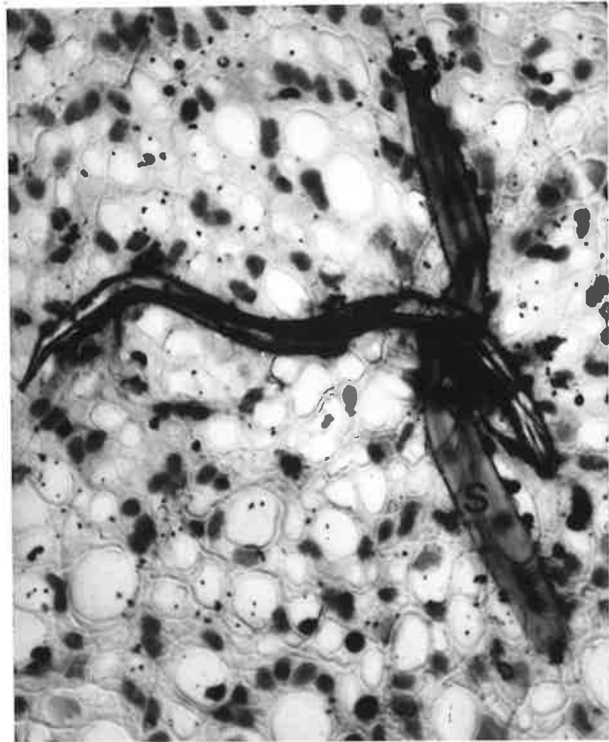

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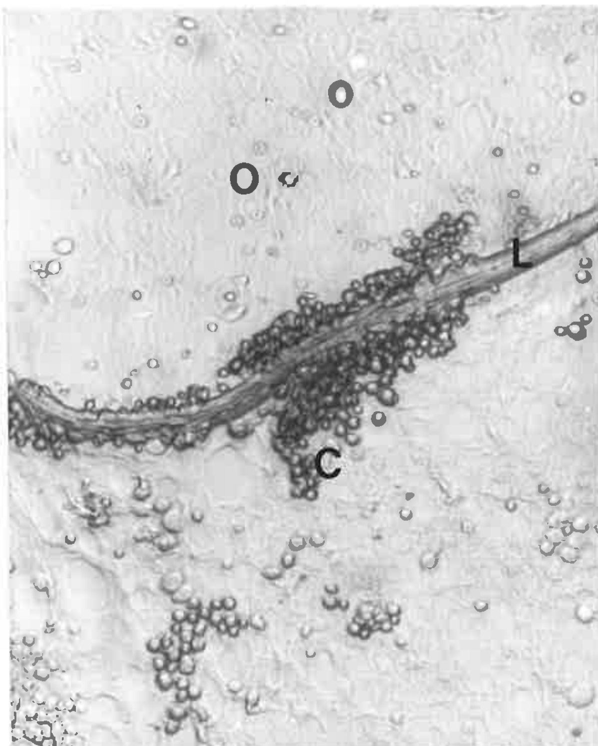

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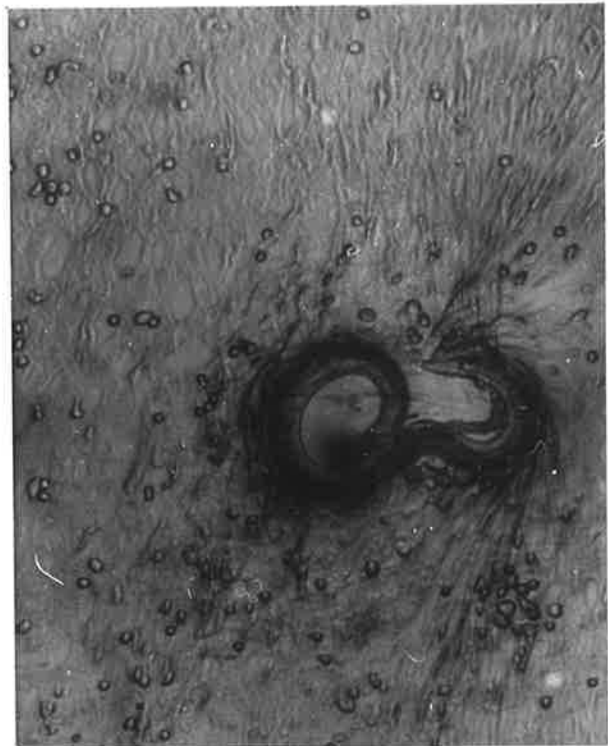

4

FIGURE 6.3

1\. and 2. Omenta from immune and normal mice 3 hr after the intraperitoneal injection of the third stage larvae. Note larvae inside the cell mass and attached to the omentum of immune mouse whilst only few cells adhered to the larvae on the omentum of the normal mouse.

L = Larvae

CM = Cell mass

O = Omentum

Wright's stain 100X

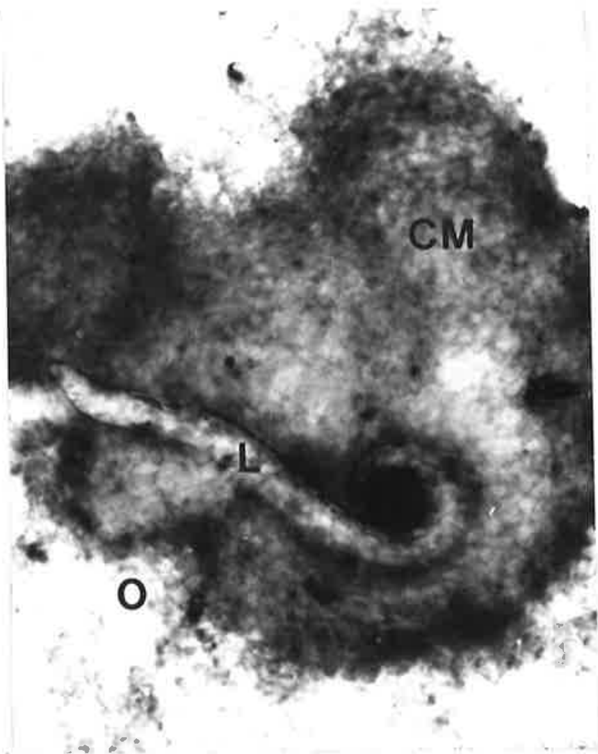
3\. and 4. Histological section of the omenta from immune and normal mice 3 hr after the peritoneal injection of the third stage larvae. Note the cellular granuloma around the third stage larvae and the accumulation of cells on the omenta of immune mouse. The omentum from normal mouse appeared normal.

L = Larva

O = Omentum

Haematoxylin-eosin 256X

Figure 6.3



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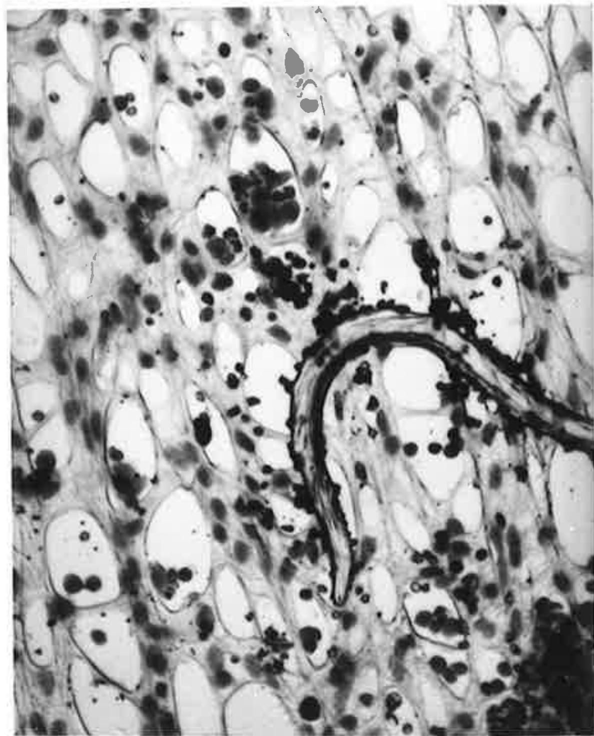

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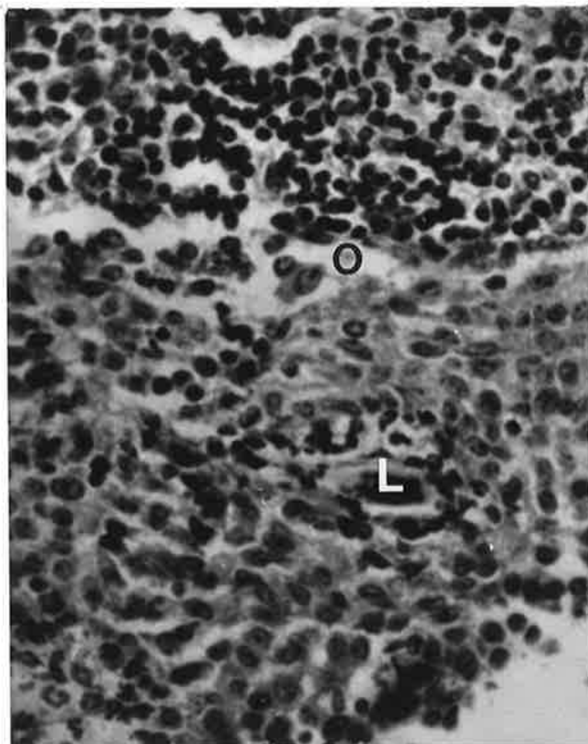

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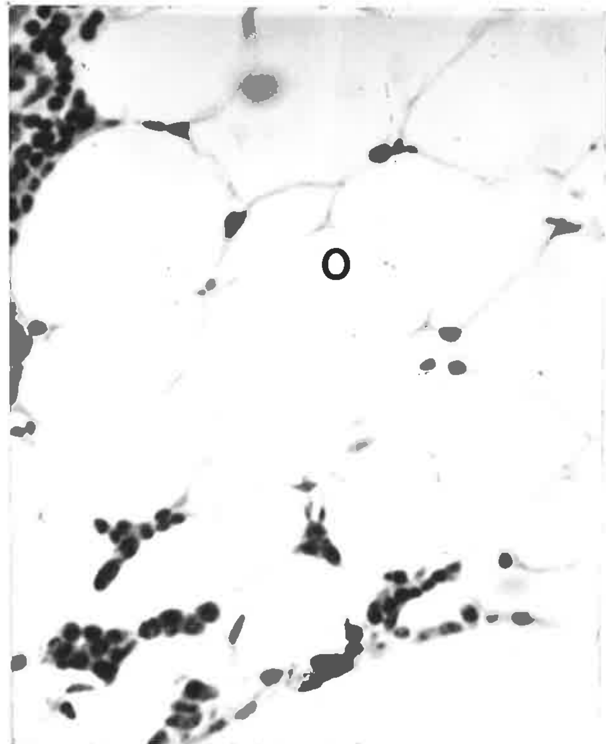

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FIGURE 6.4

1. and 2. Histological section of the omenta from immune and normal mice 12 hr after the peritoneal injection of the third stage larvae. Note the polymorphonuclear cells infiltrating around the third stage larvae on the omentum of immune mouse. The larvae were free from cells on the omenta of normal mouse.

L = Larvae

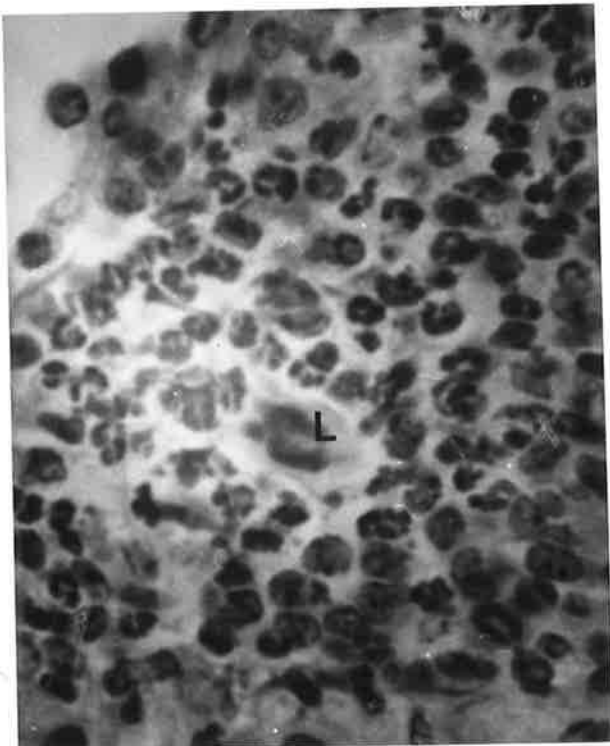
1. Haematoxylin-eosin 640X
2. Haematoxylin-eosin 256X

3. and 4. 24 hr after the peritoneal injection of the third stage larvae in immune and normal mice respectively.

L = Larva

Haematoxylin-eosin 256X

Figure 6.4



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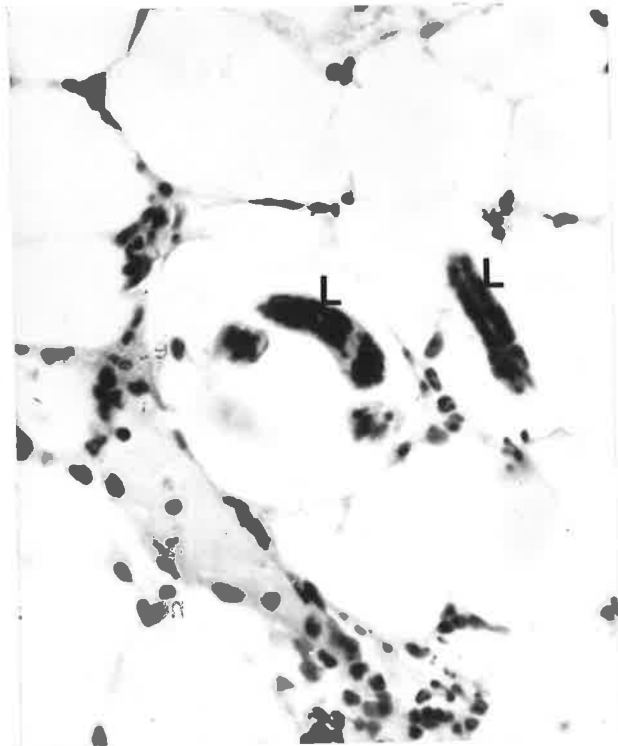

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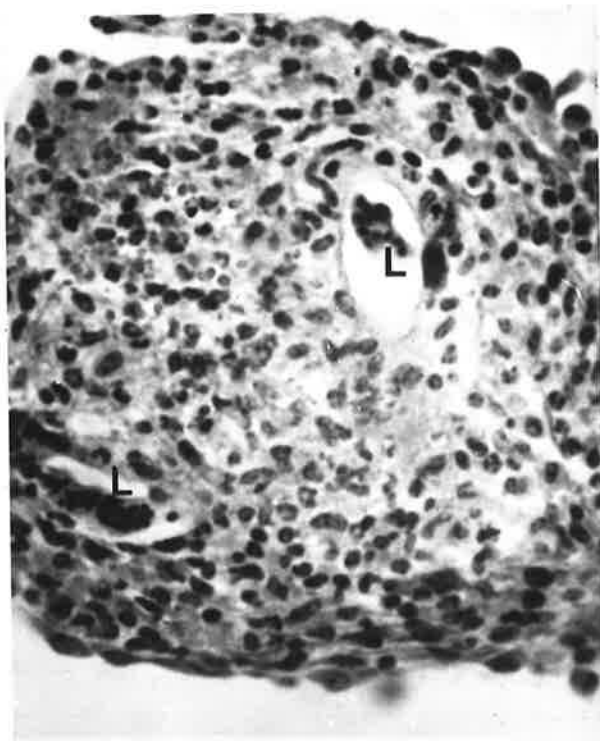

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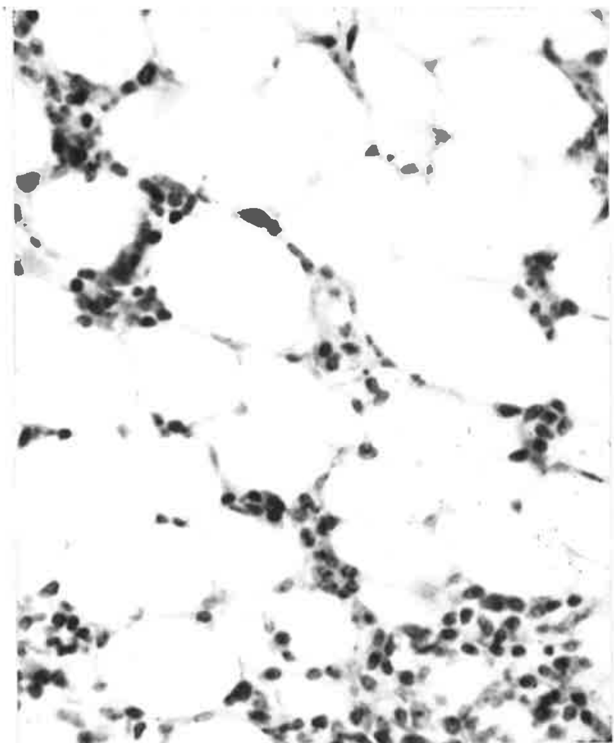

4

CHAPTER 7

THE *IN VITRO* EFFECTS OF MOUSE LYMPHOID CELLS AND PERITONEAL EXUDATE CELLS ON THE INFECTIVITY OF EXSHEATHED THIRD STAGE LARVAE OF *Nematospiroides Dubius*

1. INTRODUCTION

Previous data has shown that immunity to this infection may be transferred passively using suspensions of spleen cells and mesenteric lymph node cells from immune animals. Likewise it has been shown that *in vivo*, using a millipore chamber, peritoneal exudate cells of immune animals has a marked effect on the infectivity of the third stage larvae. However none of these *in vivo* experiments defined the cell type responsible for the inactivation of the parasite. Moreover the fact that immunity could not be transferred passively with serum from immune animals did not rule out the possibility that specific antibody was an important pre-requisite for allowing sensitised 'activated' cells to recognise and to come into intimate contact with the parasite. The following *in vitro* experiments were designed in an attempt to identify the cell type recorded in the inactivation of the parasite and to see if specific antibody played any role in this reaction.

2. CYTOTOXIC EFFECT OF SPLEEN CELLS AND PERITONEAL EXUDATE CELLS ON ISOTOPICALLY LABELLED LARVAE OF *Nematospiroides dubius*

Experiments were designed to measure the cytotoxicity of cells

from immune animals for the larvae of N. dubius by using isotopically labelled exsheathed third stage larvae, and measuring the release of the label after exposure of the larvae to these cells under varying conditions. It suffices to say that these experiments were a failure due to the fact that though the larvae could be labelled with both ^{51}Cr and ^{32}P the label was released into the medium very rapidly in the absence of cells. In view of this, in all the following experiments larvae were recovered after contact with cells under varying conditions, and their infectivity measured after feeding to normal mice.

3. THE IN VITRO EFFECT OF SPLEEN CELLS FROM IMMUNE MICE IN THE PRESENCE AND ABSENCE OF ANTISERUM ON THE INFECTIVITY OF EXSHEATHED THIRD STAGE LARVAE OF *Nematospiroides dubius*

Immunised and non-immunised F1 hybrid mice were used as the donors for cells and serum. Spleen cells (1×10^8) from either normal or immune mice in 0.4 ml of 199 medium were pipetted into the wells of a haemagglutination tray. To each well 220 exsheathed larvae in 0.2 ml of 199 medium were added. The experiment was arranged such that there were five rows of five wells containing spleen cells. Two of these rows contained spleen cells from immune animals whilst the other three contained spleen cells from normal mice. To one of the rows of spleen cells from immune mice 0.1 ml of antiserum was added, to the other 0.1 ml of 199 medium. The rows containing normal spleen cells were treated as follows. One row had 0.1 ml of antiserum added to it, the second row

0.1 ml of serum from normal mice and the third 0.1 ml 199 medium. As a control series a further 6th row of wells was set up in which larvae were incubated in 199 medium alone. The tray was then incubated at 37°C in a humid incubator in the presence of a 5% CO_2/air mixture for 2 hr. Following incubation the contents of each well were fed orally to mice which had been divided into six groups of five. Five days after oral challenge the mice were killed and the number of cysts in the wall of the intestine counted. The data given in Table 7.1 show that incubating larvae with spleen cells from immune mice affects their infectivity and the activity of these cells is enhanced in the presence of antiserum. In contrast spleen cells from normal mice had no effect on the larvae even in the presence of antiserum. These experiments were repeated using longer periods of incubation of up to 24 hr but in each case the results were similar to those obtained after 2 hr incubation.

4. THE EFFECT OF MESENTERIC LYMPH NODE CELLS FROM THE MOUSE ON THE INFECTIVITY OF THE LARVAE

Mesenteric lymph nodes were collected from immunised mice 6 days after the last immunisation as previously described. Mesenteric lymph nodes were collected also from normal mice. The cells were prepared as described in Chapter 2, Section 6.2. The experimental design was essentially the same as above. The larvae (230) were added to each well and the mixtures incubated for 3 hr at 37°C. The six rows consisting of six wells in each row were set up as follows; row 1, 6 x 10^7

TABLE 7.1 The numbers of cysts recovered from mice which received exsheathed third stage larvae of N. dubius preincubated in spleen cell suspensions from either normal or immune mice in the presence of either antiserum or normal mouse serum.

	Group 1* Cells from immune mice and anti-serum	Group 2 Cells from immune mice alone	Group 3 Cells from normal mice and anti-serum	Group 4 Cells from normal mice and normal serum	Group 5 Cells from normal mice alone	Group 6 199 medium
	Number of Cysts					
	89	119	195	199	195	209
	63	159	171	209	199	217
	96	154	219	224	223	218
	66	142	191	187	227	205
	73	126	207	209	185	250
Mean	77.4	140.0	196.6	205.6	205.8	219.8
S.d. of mean	14.5	17.3	18.0	13.7	18.3	17.7

P value "U" test

1 vs 2 P = 0.005
1 vs 3 P = 0.005
1 vs 6 P = 0.005
2 vs 3 P = 0.005
2 vs 5 P = 0.005
2 vs 6 P = 0.005
3 vs 4 vs 5 vs 6 P > 0.05

* each figure in the columns indicate cysts recovered from a mouse.

mesenteric lymph node cells from immune mice in 199 medium, row 2, 6×10^7 mesenteric lymph node cells in 199 medium and 0.1 ml of antiserum, row 3, 6×10^7 mesenteric lymph node cells from normal mice and 199 medium, row 4, 6×10^7 lymph node cells and 0.1 ml of antiserum, row 5, 199 medium alone and finally row 6, 6×10^7 spleen cells from immune mice and 199 medium. After 3 hr incubation the contents of each well were fed orally to 6 groups of mice; each group represented one row of wells. The mice were killed 6 days after challenge and the cysts counted. The results presented in Table 7.2 show that mesenteric lymph node cells from immune animals damaged the larvae as measured by loss in infectivity and this reaction was enhanced in the presence of antiserum. Lymph node cells from normal animals had no effect on the larvae even in the presence of antiserum. Longer periods of incubation of up to 24 hr did not enhance the effect of the cells from immune animals on the larvae.

These experiments established that cells from the spleen and mesenteric lymph nodes of immune animals could damage exsheathed third stage larvae and this reaction was increased in the presence of antiserum. The population of immunologically reactive cells in the spleen and lymph node are mixed and consist of both T and B lymphocytes and macrophages. Both T lymphocytes and macrophages have been shown to be cytotoxic and more recently a less well defined cell known as the K or A cell (Allison, 1972a). The K or A cell is present in the spleen, in

TABLE 7.2. The numbers of cysts recovered from mice which had received 230 exsheathed third stage larvae of *N. dubius* which had been preincubated with mesenteric lymph node cells from either normal or immune mice, in the presence or absence of antiserum.

	Group 1 Cells from immune mice alone	Group 2 Cells from immune mice and anti-serum	Group 3 Cells from normal mice alone	Group 4 Cells from normal mice and anti-serum	Group 5 199 medium	Group 6 Spleen cells from immune mice alone
			Number of cyst recovered			
	105	35	179	177	212	152
	156	141	243	237	243	146
	157	98	189	190	244	145
	146	44	175	176	201	104
	162	112	234	209	215	139
	148	137	191	220	217	141
Mean	145.7	94.5	201.8	201.5	222.0	137.8
S.d. of mean	20.8	45.6	29.2	24.7	17.5	17.2

P value "U" test

1 vs 2 P = 0.01
1 vs 3 P < 0.005
1 vs 5 P < 0.005
1 vs 6 P > 0.05

2 vs 5 P < 0.005
2 vs 6 P < 0.025
5 vs 6 P < 0.005
3 vs 4 vs 5 P > 0.05

lymph nodes and in the peritoneal cavity. Allison has suggested that this cell may represent 10% of the cells in the peritoneal cavity which are capable of adhering to glass, the remaining 90% being typical macrophages. The experiments below were designed in a attempt to define the cell type involved in damaging the parasite.

5. THE EFFECT *IN VITRO* OF THORACIC DUCT LYMPHOCYTES ON THE INFECTIVITY OF EXSHEATHED THIRD STAGE LARVAE OF *Nematospiroides dubius*

Mice (F1 hybrids) were immunised as before and thoracic duct lymphocytes, of which more than 90% are T lymphocytes were collected according to the method in Chapter 2 Section 6.3. The collected cells were washed twice in centrifugation with 199 medium and finally resuspended in the same to give 5 x 10^6 cells/ml. Aliquots of 0.5 ml of this suspension were then added to roller tubes. To one series of tubes 0.1 ml of antiserum was added. A series of control tubes were set up containing 0.5 ml of 199 medium only. All tubes were then inoculated with exsheathed larvae and the mixtures, after gassing with a 5% CO_2/air mixture, rotated slowly for 4 hr at 37°C. After incubation the contents of each tube was fed orally to three groups of mice. The recovery of cysts on day 6 (Table 7.3) indicates that thoracic duct lymphocytes from immune mice had some effects on the infectivity of the larvae. This experiment was repeated several times with varying times of incubation up to 24 hr with similar results.

As mentioned before the two other cell types that have been

TABLE 7.3 The numbers of cysts recovered from mice which had received 180 exsheathed third stage larvae of *N. dubius* which had been preincubated in a suspension of thoracic duct cells from immune mice with sera from either immune or normal mice or in 199 medium.

	Group 1 Cells from immune mice and anti-serum	Group 2 Cells from immune mice and normal serum	Group 3 199 medium
	Number of cysts recovered		
	160	110	153
	137	141	217
	98	139	146
	114	153	162
	137	112	229
	167	128	182
	94	121	162
	125	119	159
Mean	127.1	127.9	168.8
S.d. of mean	23.1	13.8	26.3

P value from "U" test

1 vs 3 P < 0.001

2 vs 3 P < 0.001

1 vs 2 P > 0.05

implicated in cytotoxic reactions are the macrophages and the K or A cells both of which are present in the peritoneal cavity and adhere strongly to glass. The peritoneal cavity also contains lymphocytes. However in the mouse strains investigated in this study these cells constituted only about 10-15% of the total population, and because they did not adhere to glass they could be separated from the other two cell types.

6. EFFECT *IN VITRO* OF PERITONEAL EXUDATE CELLS FROM IMMUNE ANIMALS ON THE INFECTIVITY OF EXSHEATHED THIRD STAGE LARVAE

Experiments *in vivo* had established that peritoneal cells from normal animals had little or no effect on the infectivity of the larvae even in the presence of serum from immunised animals. In view of these results experiments were carried out *in vitro* with cells harvested from immunised mice. The cells were collected 7 days after the last intravenous injection of living larvae. The monolayers were prepared in Leighton tubes as described in Chapter 2 section 6.4. After the addition of 0.4 ml of tissue culture fluid to each tube, the cells were allowed to settle and spread, then divided into three groups of six. To each tube in Group 1 was added 0.1 ml of antiserum, to Group 2, 0.1 ml of serum from normal mice, whilst to Group 3, 0.1 ml of medium. A fourth group of six tubes contained tissue culture medium only. To each tube was added 100 exsheathed larvae and after gassing with a 5% CO_2/air mixture, the cells and larvae were incubated for four hr at 37°C. After

incubation the Leighton tubes were shaken vigorously manually so that all the larvae were detached from the cells.

Normal mice were divided into four groups each of six animals representing the four groups of Leighton tubes. The suspended larvae were then fed orally to the respective mice. All animals were killed five days after challenge and the number of cysts counted. Results given in Table 7.4 show that peritoneal cells harvested from immunised mice had a marked effect on the infectivity of the larvae. This effect was not enhanced in the presence of specific antiserum. These experiments were repeated using varying times of incubation of up to 48 hr. The results of these experiments were essentially similar to those obtained following incubation of the larvae with cells for four hr at 37°C.

7. SYNERGISTIC EFFECT OF ADHERENT AND NON-ADHERENT PERITONEAL CELLS ON THE INFECTIVITY OF EXSHEATHED THIRD STAGE LARAVE

Previous experiments had shown that non-adherent cells such as thoracic duct lymphocytes could damage the larvae, though their effect was not as pronounced as those obtained using adherent cells. It was possible that the two cell types may cooperate in a synergistic fashion. In view of this possibility the following experiments were designed. Mice were immunised three times with living larvae as before and the peritoneal cells harvested 14 days following the last injection. Monolayers consisting of 2 x 10^6/cells per Leighton tube were prepared

TABLE 7.4 The numbers of cysts recovered from mice which had received exsheathed third stage larvae of *N. dubius* which had been preincubated with monolayers of peritoneal cells from immune mice at 37°C for 4 hr.

	Treatment of Group			
	Cells from immune mice and anti-serum	Cells from immune mice and normal serum	Cells from immune mice alone	Culture medium
	Numbers of cyst			
	60	53	68	84
	43	50	54	88
	37	45	63	89
	34	49	41	100
	33	44	48	61
	32	31	38	79
Mean	39.8	45.3	52.0	83.5
S.d. of mean	10.7	7.8	11.9	13.0

P value "U" test

1 vs 2 vs 3	$P > 0.05$
1 vs 4	$P < 0.005$
2 vs 4	$P < 0.005$
3 vs 4	$P = 0.005$

TABLE 7.5 The numbers [illegible] cysts recovered [illegible]om mice which had received exs[illegible]ned third sta[illegible] larvae of *N. dubius* which had bee[illegible] [illegible]eincubated [illegible]lherent or suspension culture of pe[illegible] [illegible]al cells [illegible]mmune mice.

	[illegible]nt of Gr[illegible]		
	Group 1 Adherent cells from immune mice	Group 2 Suspended cells from immune mice	Group 3 Culture medium
		Numbers of cyst	
	215	182	245
	157	156	288
	177	149	216
	164	139	229
	190	187	239
	193	156	243
	147	170	247
	187	208	226
	237	180	235
Mean	185.2	169.7	240.9
S.d. of mean	28.4	22.7	20.3

P value "U" test

1 vs 2 $P > 0.05$

1 vs 3 $P < 0.001$

2 vs 3 $P < 0.001$

as previously described. A similar number of cells were added to roller tubes which had been previously siliconised. Such treatment prevented the cells from adhering to the class. Exsheathed larvae (250) were added to each Leighton tube and roller tube, and, after gassing the Hanks buffered salt solution with a 5% CO_2/air mixture, the tubes were incubated at 37°C for 12 hr. The roller tubes were turned on a rolling drum at a rate of 0.4 cycles/min. The extended incubation time of 12 hr was chosen to allow optimal opportunity for cells in the roller tube to contact and adhere to the larvae. A control series of tubes contained tissue culture medium without cells plus a similar number of larvae. After incubation the controls of each tube were harvested and fed to three groups of mice representing the three situations described above. Data given in Table 7.5 show once more that peritoneal cells from immune animals are able to damage the larvae as measured by a loss in infectivity, and that non-adherent cells in the population do not enhance this effect.

8. THE ACTION OF TRYPSIN ON PERITONEAL EXUDATE CELLS FROM IMMUNE MICE

The above results indicate that the action of peritoneal exudate cells from immune mice is not enhanced in the presence of specific antiserum. This is in contrast to those results obtained from experiments using spleen cells and mesenteric lymph node cells previously reported in this chapter.

The results of investigations in other related fields have

indicated that the ability of these cells to operate in the absence of added specific antibody may be related to specific trypsin-labile receptors on the membrane. In order to test this possibility monolayers of peritoneal exudate cells were prepared from immunised mice. The monolayers were then divided in three groups. Two groups were treated with 0.05% trypsin at 37°C for 1 hr. After treatment with the enzyme the monolayers were washed three times _in situ_ with tissue culture medium. Finally 0.6 ml of medium was added to one group of tubes. To the other trypsin treated monolayer was added 0.5 ml of medium and 0.1 ml of specific antiserum obtained from the same mice from which the cells had been collected. The third non-treated group of cells was washed _in situ_ also with three changes of medium, and 0.5 ml of fresh medium and 0.1 ml of serum from normal mice added. Finally into each tube was placed 200 infective larvae and the monolayers were incuabated at 37°C for 12 hr. A fourth group of control tubes contained larvae and tissue culture medium only. After incubation the larvae were harvested as in previous experiments and fed to four groups of mice representing the four series of tubes. The results given in Table 7.6 showing the number of cysts recovered on day 5 after oral challenge indicate that trypsin destroyed completely the ability of the exudate cells to damage the parasite. However the addition of antiserum restored this capacity. The results of previous experiments (see Chapter 5) indicate that the production of specific antibody alone was not sufficient to elicit the best immunity to infection. The experiments given in Chapter 6 and this

TABLE 7.6 The numbers of cysts recovered from mice which had received exsheathed third stage larvae of *N. dubius* which had been preincubated in trypsinised cells, trypsinised cells plus immune serum or non-trypsinised peritoneal cells from immune mice plus normal mouse serum for 21 hr.

	Treatment of Group			
	Group 1 (Trypsinised)	Group 2 (Trypsinised plus antiserum)	Group 3 (non-trypsinised plus normal serum)	Group 4 Hank's BSS
	151	130	145	158
	181	152	128	192
	193	154	137	177
	192	110	132	150
	216	126	107	183
	181	105	145	160
	161	109	116	167
	204	135	147	204
	205			
Mean	187.1	127.6	132.1	173.9
S.d. of mean	21.1	19.0	14.6	18.5

P value "U" test

1 vs 2 $P < 0.001$
1 vs 3 $P < 0.001$
2 vs 3 $P > 0.05$
1 vs 4 $P > 0.05$
2 vs 4 $P < 0.001$
3 vs 4 $P < 0.001$

chapter show that cells obtained from immunised animals have an altered capacity to deal with this parasite when compared with those obtained from normal mice. However the results of the experiment using peritoneal exudate cells that had been treated with trypsin indicate that specific antibody may play an important role in directing the activity of the cell. If this were so it might be possible to 'activate' cells by other means, and, providing antibodies specific for the parasite were present, these cells may function in a comparable manner to those obtained from mice which had been immunised with the living larvae. To test this hypothesis the following experiment was designed.

9. EFFECT OF PERITONEAL EXUDATE CELLS FROM MICE GIVEN LIVING *S. enteritides* IIRX ON LARVAE OF *Nematospiroides dubius*

As stated in Chapter 4, peritoneal exudate cells from mice immunised with *S. enteritides* IIRX, show both enhanced bactericidal properties and cytotoxicity to certain tumour cells (Ashley and Hardy, 1973). In bacteria the enhanced bactericidal properties may be demonstrated only in the presence of antibody specific to the test organism.

A group of mice were injected intraperitoneally with 1 x 10^5 *S. enteritidis* IIRX. Eight days after this challenge the cells were harvested from the peritoneal cavity and monolayers prepared as

TABLE 7.7 The numbers of cysts recovered from mice which had received 250 exsheathed third stage larvae of N. dubius which had been preincubated in monolayers of the peritoneal cells from IIRX, infected mice in the presence or absence of specific antiserum to N. dubius or from normal mice.

	Group 1 Cells from IIRX infected mice	Group 2 Cells from IIRX infected mice and antiserum	Group 3 Cells from normal mice and antiserum
	Numbers of Cyst		
	232	173	204
	239	141	206
	232	139	220
	208	141	222
	216	156	231
	244	165	211
	224	142	195
	204	149	221
	213		
Mean	223.6	150.8	213.8
S.d. of mean	14.1	12.7	11.8

P value "U" test

1 vs 2 $P < 0.001$

2 vs 3 $P < 0.001$

1 vs 3 $P > 0.05$

previously described. The tubes were divided into two series. To one series tissue culture medium was added in the absence of specific antiserum whilst to the other series 0.1 ml of specific antiserum was added. The final volumes in each tube was 0.6 ml. Finally 250 exsheathed infective larvae were placed into each tube and the cell cultures incubated at 37°C for 12 hr. A series of control tubes contained peritoneal exudate cells from normal mice in the presence of the same quantity of antiserum and number of larvae. Following the period of incubation the larvae were harvested from the tubes and fed to three groups of mice. Data given in Table 7.7 on the number of cysts recovered on the fifth day after the oral challenge show that adherent peritoneal exudate cells are capable of inactivating the larvae but only in the presence of specific antibody. In contrast, exudate cells from normal mice are ineffective. These data will be commented on further in the discussion.

10. CONCLUSIONS

The results given indicate that the resistance of immunised mice to this infection is dependent on the altered activity of certain cells which are present in the spleen, mesenteric lymph nodes and peritoneal cavity. The activity of the cells in the spleen and lymph nodes is enhanced in the presence of specific antiserum, though they can function in its absence.

The addition of specific antibody to cells from the peritoneal cavity doesn't enhance the damaging effect that these cells have on the larvae. Experiments with trypsin suggest that the activity of these cells to damage the parasite is related to the presence of trypsin labile receptors on the cell membrane. These receptors are probably cytophylic antibody since one can restore ability of trypsin treated cells to damage the parasite if specific antibody is added to the system. Peritoneal exudate cells from mice that have been given Salmonella enteritides IIRX are capable of damaging the parasite but only in the presence of specific antiserum.

These results will be discussed in greater detail in Chapter 8.

CHAPTER 8

DISCUSSION

The present study has shown that the mouse strains used in this investigation are equally susceptible to infection with the parasite as judged on the percentage of cysts and adult worms recovered following challenge with a known number of larvae. However it is clear from the LD50 studies that mice of the Balb/C strain cannot carry as heavy a worm burden as Swiss White mice. The LD50 for the former strain was 117 whereas for the latter it was 500. It was surprising to find that new born mice were resistant to infection than were adult animals. This is in contrast to what has been reported for certain other nematode infections where the young are particularly susceptible to infection (Jarrett, 1971; Jarrett and Urquhart, 1971). However Dobson (1961) has reported also that 10-day-old Swiss White mice are more resistant to infection with *N. dubius* that are adult mice. The reason for their increased resistance is not known, though it is possible that the physiological environment of the small intestine in new born mice is such that few of the larvae are able to develop to the adult stage. The importance of an unfavourable physiological environment should be borne in mind when later in the discussion one considers the transfer of immunity from immune mothers to their offspring.

It is clear from earlier studies (see Chapter 1 Section 3.5) that mice previously infected with *N. dubius* are immune to re-infection. However the mechanisms involved in this acquired immunity are obscure.

In the present study an attempt has been made to delineate the importance of specific antibody and the co-operation of cells in this acquired resistance to infection. The data given in Chapter 4 indicates that mice immunised intravenously with living third stage larvae are immune to reinfection. The results show that the immunity is expressed against the infective larval stage and not against the adult worm. This conclusion is based on the fact that whilst there is a highly significant reduction in the number of larvae encysting in immune mice as compared with that observed in normal mice given a known number of larvae, the number of adult worms recovered from immune mice correlates well with the number of larvae that have encysted.

It was observed also that mice immunised with third stage larvae produced circulating antibodies. This is in contrast to the findings of Panter (1967). However the results on antibody titre following immunisation with third stage larvae reported in this thesis are by no means quantitative. They rely on the adsorption of antigens from an extract of adult worms onto sheep erythrocytes. It is more than likely that the mice immunised as above produced specific antibodies to antigens that were not adsorbed to the erythrocytes. Nevertheless the data shows the presence of circulating antibody in immune mice. Following the demonstration of circulating specific antibody, attempts were made to transfer immunity passively with serum from immune mice. It suffices to say that all these attempts failed despite the fact that in some instances antiserum was administered for quite long periods of time. Passive

immunisation with antiserum has been successful in protecting the host against certain nematodes (Jarrett and Urquhart, 1971; Sinclair, 1970) though other workers have reported consistant failure to achieve immunity by this means using other species. (Dineen and Wagland, 1966; Larsh, 1967). One might argue that the protective antibody was associated with a specific class of immunoglobulin molecules such as IgA or IgE. Both these classes are present in low titre in the serum. The IgA class of immunoglobulin is present in high titre in mucous secretions and colostrum whilst the IgE class has a high affinity for the membranes of certain cells such as mast cells. If for example the 'protective' antibody was associated with the IgA class of immunoglobulin we could account for the passive transfer of immunity from immune mice to the neonate. It is clear from these experiments that immunity is transferred after birth and not whilst the animal is in-utero. However this does not appear to be the case since neonates born of normal mothers that were given serum from immune mice during pregnancy were protected from infection. Thus it would appear in this case that the protective antibodies are present in the serum.

These results are difficult to explain, circulating antibody appears not to protect, adult mice against infection whilst it does protect the neonate. Earlier data had shown that new born mice are much more resistant to this infection that are adult animals, and it was suggested earlier in this discussion that the physiological environment did not favour development of the infective larvae. In such a situation

specific antibody alone may be sufficient to tip the scales further in favour of the host. There is some precedence for the arguement although it does not offer an explanation of the effector mechanism through which antibody operates. The guinea pig is an abnormal host for the nematode parasite _T. colubriformis_, the normal host being the sheep. In the guinea pig the percentage of infective larvae which reach maturity is very low. In such a host parasite relationship as this, one can transfer immunity passively with serum (Connan, 1972).

Whilst these studies did not rule out the possibility that Cytophilic antibody may be playing an important role in determining the resistance of immunised mice to reinfection, the failure to transfer immunity passively with serum led one to examine the role of cells as an important effector mechanism in determining resistance.

It was found that immunity may be transferred passively with either spleen cells or mesenteric lymph node cells obtained from immune animals. Since the cells obtained from the spleen and mesenteric lymph nodes are a mixed population consisting of macrophages and T and B lymphocytes it was not possible to define the cell type which may be playing the central role in the expression of immunity. For that reason cell transfer experiments were carried out using thoracic duct lymphocytes and peritoneal exudate cells. Thoracic duct lymphocytes are a rich source of T lymphocytes. These cells have been shown to play an important role in the rejection of tissue grafts (see Gowans and McGregor, 1965; Wilson and Billingham, 1967) and may be cytotoxic to certain tumour cells if

obtained from animals previously immunised against that tumour (Brunner *et al.*, 1970). Peritoneal exudate cells were chosen as a source of macrophages. In the mouse strains used in this particular study more than 90% of the peritoneal exudate cells were typical macrophages. These cells have been shown to be cytotoxic to various tumour lines if they have been harvested from animals immunised against the tumour. (Granger and Weiser, 1964; Evans and Alexander, 1970, 1971, 1972). Earlier in the thesis it was suggested that the immune mechanism operating against tumour cells and tissue grafts may be similar to those operating against metazoan parasites.

The results of these experiments showed that immunity may be transferred with thoracic duct lymphocytes but not with peritoneal exudate cells. These results suggested a central role for the T lymphocyte and support the data of Dineen and Adams (1971) who found that immunity in guinea pigs to *T. columbriformis* infections could be transferred adoptively with sensitised thoracic duct lymphocytes. The T lymphocyte circulates through the body constantly and it was felt that perhaps the failure to transfer immunity with peritoneal exudate cells might be due to the fact that insufficient number of cells were present at the site of infection, i.e. the small intestine. In order to investigate this more carefully larvae were injected into the peritoneal cavity of both immune and normal mice and recovered after a period of time. It was found that a significant percentage of the larvae had lost

their infectivity. Within a very short time larvae injected into the peritoneal cavity of immune mice became surrounded by large number of cells, which were mainly macrophages. These cells adhered strongly to the cuticle and could be removed only by treatment with trypsin. Experiments involving the implantation of millipore chambers containing larvae showed that damage to the parasite required contact of the cells with the cuticle of the larvae. Examination of the cells migrating into the chamber revealed that they were mainly macrophages. Possibly the most significant finding in the work with millipore chambers resulted from studies using millipore chambers containing antiserum and larvae that were planted into the peritoneal cavities of normal mice. Large number of macrophages migrated into these chambers and were found adhering to the larvae in a large mass. However in contrast to the situation observed in immune mice the larvae were not damaged. In control chambers containing normal mouse serum few cells had migrated into the chamber. These results are important for two reasons. The first is that specific antibody enables the cells to recognise the parasite and secondly they suggest that damage to the larvae can occur only if the cells have been altered or 'activated' in some way. If an altered state in the activity of the cell is required before damage to the larvae can occur this would explain our failure to transfer immunity passively with serum.

The ability to transfer immunity with either spleen cells, mesenteric lymph node cells and thoracic duct cells could be explained in

two ways. The first is that the transferred cell population contains activated cells which attack the parasite and the second is that the population of transferred cells contains sensitised cells which are able to activate host cells. Mackaness (1970) has suggested that 'activation' of macrophages requires firstly the interaction of specifically committed lymphocytes with antigen. The results of *in vitro* experiments using population of cells from the spleen and mesenteric lymph nodes indicate that these populations contained cells capable of damaging the parasite. The effectiveness of these cells was enhanced in the presence of specific antibody. Thoracic duct lymphocytes by contrast were not as effective as those two latter populations. The fact that one can demonstrate cells capable of damaging the parasite does not rule out the possibility, that in recipients receiving these populations specifically committed lymphocytes by the mechanism suggested by Mackaness (1970) are able to activate host cells which participate in attacking the parasite.

Similar experiments *in vitro* carried out with peritoneal exudate cells show that these cells also damage the larvae. This property is not enhanced in the presence of specific antiserum. Treating the cells with trypsin destroys their ability to attack the larvae. However this property is restored in the presence of specific antiserum. This would suggest that the trypsin labile factors in the surface of the peritoneal exudate cells are cytophilic antibodies, and would also explain the ability of spleen cells and mesenteric lymph node cells to damage the larvae in the absence of specific antibody. The increased effect that these latter

population of cells have in the presence of specific antiserum may merely reflect the varying amounts of cytophilic antibody associated with the cell population. These results indicate that specific antibody is important in directing the activity of the cell.

This fact is supported by the results of experiments involving *Salmonella enteritidis* IIRX. Other workers have shown that mice pre-treated with *S. enteritidis* IIRX, are resistant to various pathogenic bacteria and are immune also to certain tumour lines such as the Ehrlich Ascites tumour. (Ashley and Hardy, 1973). The basis for this immunity is related to an activation of the macrophages.

Earlier in the thesis experiments are described which indicate that mice immunised with a living vaccine of *S. enteritidis* IIRX show a marginal degree of protection. In view of the above results, experiments were designed to see if peritoneal exudate cells harvested from these treated mice could damage the larvae. The data obtained from these investigations show quite clearly that when larvae are added to such peritoneal cells in the presence of specific antiserum there is a significant loss of infectivity. In the absence of antiserum the larvae are not damaged.

In conclusion the data presented in the thesis supports the hypothesis that immunity to *N. dubius* infections in mice requires the co-operation between an activated cell and specific antibody. The type of cell has not been clearly defined though results of *in vivo* studies

in which larvae were injected into the peritoneal cavity would suggest that cell is or resembles a macrophage. It is possible that the important cell involved in the cytotoxic action is the recently described K or A cell (Allison, 1972a, b) which would be present to a varying degree in all the cell populations studied, and would be difficult to identify from the macrophage.

Further if the above is a general phenomenon and one is thinking of producing efficient vaccines which protect individuals against helminth infections, one must aim at one which produces both activated cells and specific antibody.

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