# THE DEVELOPMENT OF RESISTANCE AND IMMUNE RESPONSES IN MICE TO INFECTION WITH *NEMATOSPIROIDES DUBIUS*

by

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# ABSTRACT

This investigation examined the changes in the immune status of the host and the development and mechanisms of resistance to infection by the murine gastrointestinal nematode, Nematospiroides dubius (Heligmosomoides polygyrus).

The initial studies showed that there were variations in the ability of different inbred strains of mice and an outbred strain of mouse to develop immunity as assessed by their ability to resist a larval challenge infection, to expel adult worms and to form granulomata. The ratio of the number of cysts to the number of granulomata proved to be a very sensitive index of resistance, highlighting the differences between the inbred mouse strains. These studies also showed that female mice developed better protective immunity than male mice of the same strain. Further studies involving the development of resistance in the progeny of crosses between various strains indicated that some of the genes controlling the development of resistance were linked to the Y chromosome.

Investigations into the fate of larvae following the challenge of resistant outbred LACA mice showed that the larvae penetrated the intestinal mucosa, where they were killed. These studies also showed that the cyst stage of the life cycle alone could induce good resistance, without the presence of the adult worms. Further studies in these mice indicated that there were marked changes in the number of leukocytes in the blood and peritoneal cavity during

the course of the development of resistance. These changes were due to increased numbers of macrophages, neutrophils, eosinophils and lymphocytes. By far the most dramatic change was that of the appearance of the eosinophils in the blood and the peritoneal cavity. These appeared following the second immunising infection. The reason behind this was not clear since the first immunising infection caused an increase in the number of eosinophils in the bone marrow. Following two immunising infections there was an enormous increase in the level of $IgG_1$, in some cases up to 50-fold. This appeared to be the only isotype whose production was stimulated by infection.

Attempts were made to relate these changes to the development of immunity. It was found that a primary infection would induce partial resistance and full resistance occurred following the administration of a second dose of larvae. The development of full resistance could be correlated with the appearance of eosinophils in the peritoneal cavity and the blood stream. It was also found that the injection of $IgG_1$ isolated from the serum of resistance mice into mice following a primary infection greatly enhanced the degree of protection obtained. These results suggested that eosinophils and specific antibody of the $IgG_1$ isotype may play a role in the expression of full resistance.

Studies of the development of resistance in nude mice showed that they were unable to form granulomata nor expel

adult worms following immunisation. They could, however, develop a degree of resistance against a larval challenge, but the development of full resistance together with granuloma forming ability, adult worm expulsion and $IgG_1$ production was dependent upon the mice having a complete complement of T cells. Studies involving the transfer of serum from resistant mice to nude mice suggested that the activated macrophages of the nude mice may play a role in mediating this partial resistance to infection.

The interaction between the larvae and the immune system of the host was studied in detail. The presence of antibody directed against the cuticle of larvae and adult was demonstrated in the serum from mice resistant to reinfection. This antibody was also on the surface of peritoneal exudate cells from resistant mice. This was demonstrated by the adherence of cells to the larvae and radioimmunoassays showed that the antibody was of the $IgG_1$ isotype. It was also found that adherence of cells to the larvae was promoted by the fixation of complement by the cuticle of the larvae via the alternate pathway.

An in vitro assay to determine the effect of this cell binding upon larval infectivity indicated that the antibody on the surface of peritoneal exudate cells from resistant mice promoted their attachment to the larvae which resulted in a reduction of larval infectivity. Further studies involving the use of a goat anti-larval antisera

both *in vivo* and *in vitro* supported the concept that activated macrophages and antibody directed against antigens on the surface of the larvae are important in the expression of resistance.

These results allowed a possible mechanism of resistance to be proposed involving antibody and activated cells acting against larvae in the intestinal tissues.

S. Prowse

# 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 where reference is made in the text.

Stephen J. Prowse.

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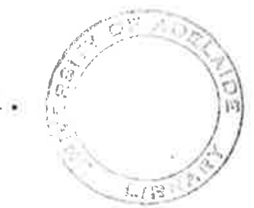

# CHAPTER I

## Introduction

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## 1. General Introduction

In most tropical and subtropical countries the human and animal inhabitants carry at least one and often multiple parasitic infections. The toll on human and animal life and health constitutes serious problems for economic and social development. The sheer magnitude of the problem is illustrated by estimates of over 20 million people infected by *Onchocerca volvulus* (W.H.O. Technical Report, No. 597), over 90 million cases of malaria occur annually in Africa alone and one million of these end in death (W.H.O. Technical Report No. 579). With the increasing number of dams being built for irrigation, there has been a dramatic increase in the number of cases of schistosomiasis. These and many other parasitic diseases result in a wide range of insidious effects on the economic status of the population. For example, a recent Pan American Health Organization Study on the impact of Malaria showed that the efficiency of rural workers was impaired to such an extent so as to cause a reduction of 10-35% in crop yields, reduced expansion of arable land and postponement and neglect of other work (W.H.O. Chronical, 1976). The adaption and transformation of subsistence farmers into settled small businesses will be greatly hindered by the inability of farmers to work at top efficiency during crucial periods such as planting and harvesting.

## 2. Nematode Infections in Man and Animals

It has been estimated that one fourth of the world's population is infected with the hookworms *Ancylostoma*

*duodenale* and *Necator americanus*. The importance of these intestinal infections as a cause of morbidity varies according to the circumstances of infection, i.e. magnitude of the worm burden, diet and concurrent infections with other parasites. In a study carried out by Gilles, Watson Willians and Ball, (1964) it was concluded that hookworm infection was the only discoverable cause of the severe anaemia in patients who came under their observation.

The most effective way to control nematode infections would be to change the social habits and economic status of the susceptible populations, but this is seldom achieved even in the more prosperous countries. In the poorer communities the introduction of sanitation had little if any effect on the level of worm infestation (Cort et al., 1929). There has been some success in preventing the spread of hookworm in regions where human excreta is used as fertilizer by storage or treatment of the excreta before use and encouraging farmers to wear shoes. For economic reasons it is impossible to oppose the use of excreta as a fertilizer (Cort, Grant and Stoll, 1926).

Nematode infections are easily eliminated by the use of relatively safe antihelminthic drugs, however the treated people quickly become reinfected upon return to, or re-exposure to infested regions (Gilles et al.,1964 ). It seems therefore desirable and necessary to control helminth infections by a combination of biological and social means. At the moment vector control in limited situations and chemoprophylaxis are the principle means of control. The major problems now arising

from these methods are the development of pesticide resistant vectors and drug resistant parasites.

Vaccination as a control measure remains a goal, so far unobtainable except for some success with irradiated larvae of Ancylostoma caninum (Miller, 1971; 1975) used for the vaccination of dogs.

There has been a considerable increase in our understanding of the immunological response of the host to nematode infections as a result of work carried out in laboratory animals. This increased knowledge will hopefully lead to the development of better methods of controlling these infections.

## 3. Examples of Mechanisms of Immunity to Helminths

### 3.1 Introduction

The responses to and mechanisms involved in immunity to a number of helminth infections have been studied in considerable detail. The following discussion illustrates common features in the responses of hosts to infection and outlines the factors likely to be involved in immunity against them. However despite extensive studies, in most cases the actual way in which the parasite is killed remains unknown.

### 3.2 Mechanisms of immunity to Nippostrongylus brasiliensis

N. brasiliensis is a nematode parasite of rats but will also infect mice. Most of the investigations into the immune responses of the host to this parasite have been carried out in the rat, although with the increasing availability of the nude mouse, studies in mice have become more common.

The adult forms of N. brasiliensis live in the lumen of the small intestine. Eggs are laid and pass out with the faeces and hatch into first stage larvae, which after two moults develop into infective third stage larvae. Under natural conditions the infective larvae penetrate the skin of the host and travel via the blood to the lungs where they undergo a fourth moult. They then pass up the trachea, are swallowed and upon reaching the small intestine undergo a further moult and develop into adult worms. At 5 - 6 days post infection, egg laying commences. The adult population remains static for about a further 5 days after which time egg output drops quickly and the majority of the adults are rapidly expelled (Jarrett and Urquhart, 1971).

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

1. Loss phase 1. The immobilization or destruction of larvae at the site of injection or on route to the intestine.
2. Plateau phase. The larvae which reach the small intestine develop to maturity and the numbers of adult worms then remains static.
3. Loss phase 2. The majority of adult worms are rapidly expelled from the intestine in an exponential fashion.
4. Threshold phase. A small residual population of adult worms survive.

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

intestine.

In 1936, Sarles and Taliaferro showed that in immune animals, some of the subcutaneously administered challenge dose remained trapped at the site of injection. More recently Love, Kelly and Dineen, (1974) demonstrated in immune rats, a 50% reduction in the numbers of larvae migrating from the lungs to the oesophagus and a delay of those actually reaching the oesophagus. Only a small percentage ( 17%) of those reaching the oesophagus actually developed into adult worms, many being expelled from the small intestine before they matured (Love et al., 1974; Love, 1975). The plateau phase is also shorter and the eventual expulsion rate of adult worms from the intestine is increased.

Early studies on immunity to this infection, carried out by Sarles and Taliaferro, (1936) showed that normal rats may be protected by the transfer of serum from hyperimmune animals. However large volumes of antiserum were needed and there was an enormous variation in the ability of different pools of antiserum to transfer protection. Similar results were obtained in 1971 by Ogilvie. It seemed that the number of infections which the serum donors had undergone was not important (Ogilvie and Jones, 1968; 1971). In contrast to this H.R.P. Miller (personal communication) has demonstrated a high level of expulsion, mediated by a number of pools of serum from immune rats. There was little variation in the ability of different pools of serum to cause expulsion.

Ogilvie, (1967) showed that antiserum from immune rats contained elevated levels of IgE (reaginic or skin sensitising) antibody directed against parasite antigens. This reaginic response has been shown to be a common feature of many helminth infections (Reviewed Zvaifler, 1976). It has also been shown by Jarret, (1972) that helminth infections have the remarkable effect of potentiating IgE responses against antigens, similtaneously administered, and unrelated to those of the parasite.

Despite the fact that reaginic type antibody is produced as a result of almost all helminth infections and the many studies into its possible role in protecting animals against infection, its true function remains unclear. It has been postulated for a number of these infections that upon contact between antigens and antibody of the IgE isotype, an anaphylactic reaction is elicited involving degranulation of mast cells and the release of vasoactive amines. It was proposed that because of these reactions the intestine becomes an unfavourable environment for the worm resulting in its expulsion from the host (Urqhart et al., 1965; Mulligan et al., 1965; Panter, 1969b; Miller, 1971 and Jarret and Urquhart, 1971). Although Jarrett and Bazin, (1977) have shown that IgE is the only immunoglobulin isotype to increase in concentration during infection the evidence for this response being involved in immunity is so far circumstantial. Indeed Jones, Edwards and Ogilvie, (1970) showed that fractions of antisera from rats, immune to N. brasiliensis, which were free of detectable IgE or IgG

reaginic antibody, still had the ability to elicit the expulsion of adult worms. It has also been shown by Jarrett and Urquhart, (1971) that IgE antibody had not appeared in the serum of infected rats at the time adult worms were being expelled from the intestine. It is possible however that the antigen fractions used to elicit the anaphylactic reaction involved in the detection of reaginic antibody did not contain antigens to which the reaginic antibodies were directed. Unsuccessful attempts were made to elicit the early expulsion of adult N. brasiliensis from the intestine of rats by the induction of severe anaphylactic shock using adult worm antigens. This suggested that anaphylaxis alone was not responsible for the expulsion of adult worms from the intestine.

However, there is evidence that specific antibody can damage adult worms in vitro. Ogilvie and Hockley, (1968) demonstrated that antibodies cause severe changes to the cytoplasm of the gut cells of adult worms by noting their reduced ability to take up radio labelled orthophosphate from the host. Furthermore, Henney, Maclean and Mulligan, (1971); Lee, (1970); Edwards, Burt and Ogilvie, (1972) and Jones and Ogilvie, (1972) have shown that immune rats had specific antibody directed against acetyl choline esterase produced by adult worms. The levels of the acetyl choline esterase isoenzymes of worms from the plateau phase of infection differ from those of worms from the threshold phase. It was suggested that the presence of specific antibody was responsible for these changes. In view of these results it

is difficult to explain why some pools of antisera cause expulsion of adult worms and other pools are ineffective. The fact that adult worms were not lost from irradiated rats following the administration of antiserum led Jones and Ogilvie, (1972) to suggest that a radiosensitive factor was required for rejection. They proposed a 2 step mechanism, firstly the damage of adult worms by antibody which is followed by a cellular dependent step.

A large number of experiments carried out by Keller and Kiest, (1971); Kelly and Dineen, (1972); Dineen, Kelly and Love, (1973) and Kelly, Dineen and Love, (1973) showed that mesenteric lymph node cells from immune animals could hasten the elimination of adult worms from normal and irradiated rats. These authors suggested that lymphocytes from immune animals may play a direct role in this process. However this work in no way supports a direct role for lymphocytes in immunity since mesenteric lymph nodes contain both thymus derived and bone marrow derived lymphocytes. Consequently, adoptive delayed hypersensitivity and antibody responses would result in these animals, upon antigenic stimulation by infection.

This rather confused situation was clarified to some extent by Ogilvie et al., (1977) who showed that immunity could be transferred to irradiated rats by thoracic duct lymphocytes from immune animals which had been depleted of Ig +ve cells. They showed also that the transfer of immune mesenteric lymph node cells did not consistently elicit the expulsion of adult worms from irradiated rats. This

inconsistency might be explained by the fact that at times the mesenteric lymph nodes did not contain sufficient T cells to cause expulsion. Since the irradiated rats were not reconstituted with bone marrow and hence unable to produce antibody it seems possible that antibody was not directly involved in the rejection of adult worms and that antibody dependent damage of adult worms probably was not a pre-requisite to expulsion. This is in agreement with the results of Jacobson, Reed and Manning, (1977) who showed that N. brasiliensis was still lost from mice which lacked the potential to produce antibody. These mice had received repeated injections of specific anti-$\mu$ chain antibody and could not produce antibody against parasite antigens as determined by anaphylactic reactions or against sheep red blood cells. Furthermore, Nawa, Parish and Miller, (1978) have demonstrated that cells from the thoracic duct lymph (T.D.L.) with no surface immunoglobulin, obtained from rats 10 days after a primary infection could cause the elimination of both normal and 'damaged' worms. Nawa and Miller, (1978) demonstrated also that the degree of expulsion was dependent upon the number of cells transferred. However these results are inconclusive since T.D.L. have the capacity to adoptively transfer both humoral antibody and cell mediated responses. However these results may lead to a re-evaluation of the 2-step theory of worm expulsion suggested by Ogilvie and Love, (1974). The role of T cell dependent responses in the expulsion of adult worms from mice has been emphasised further by the inability of nude mice to rid themselves of

worms (Jacobson and Reed, 1974, 1976; Mitchell et al., 1976).

There is also some evidence that prostaglandins may be involved in expulsion. Kelly et al., (1974) showed that N. brasiliensis adults were lost from the intestine of rats by an intraduodenal injection of prostaglandins. This observation is supported by the evidence of Richards et al., (1977) who showed that the action of prostaglandin $E_1$ on worms in vitro adversely affected their ability to re-establish in rats and caused visible structural damage. These experiments carried out in vivo and in vitro should be interpreted with great caution since prostaglandins of the E type have been shown to have major effects on immune responses by exerting control on lymphokine secretion, cytolysis, haemolytic plaque formation and lymphocyte mitogenisis, (Goodwin, Bankhurst and Messner, 1977). In experiments carried out in vitro, it should be ensured that the levels of prostaglandins used do not exceed those measured in the intestine during infection.

In summary, it appears that although antibodies, either reaginic or non-reaginic do have the ability to damage adult worms, it seems that neither the presence of antibody nor damage to worms is a pre-requisite for worm expulsion. Expulsion seems to be highly T cell dependent in both rats and mice.

### 3.3 Mechanisms of immunity to schistosome infections

Schistosoma mansoni is a trematode which infects man, monkeys and rodents. The infective larvae, called cercariae enter the blood stream by burrowing through the skin. After

moulting to become schistosomulae, they reach the lungs via the pulmonary vessels where they burrow through the alveoli causing extensive damage. From the throacic cavity the schistosomulae enter the liver and eventually the mesenteric veins where copulation and egg laying occurs. Eggs are laid 22-23 days after infection and may be seen trapped in the liver and intestinal mucosa. The longevity of the adult flukes depends upon the species of the host and ranges from 50 days to a number of years. Eggs burrow through tissues to be passed in faeces, hatch and develop into miracidea. These multiply asexually in a snail and emerge as infective cercariae.

The pathological effects of infection are enormous. The penetrating schistosomulae cause extensive damage to the alveoli and liver. The eggs laid, may become trapped in tissues causing extensive granulomatous lesions in the liver and intestinal tissue.

Schistosome infections have been widely studied in monkeys and rodents. These animals, although abnormal hosts are quite good models for this infection in man. Animals develop immunity to the schistosomulae after several immunising infections although the adult trematodes derived from the primary exposure to cercaria are still present. The elegant experiments of Smithers, Terry and Hockley, (1969) indicated that the reason why they survive in the immune host is due to the aquisition of host derived material which masked the antigenic determinants on the surface of the parasite.

The immune mechanisms which kill the invading schistosomulae have been widely studied and probably involve interaction between antibody and a number of different cell types. A large amount of work has been done studying damage to schistosomulae both in vitro and in vivo. Sher, McIntyre and Von Lichtenberg (1977) have shown that S. mansoni infection in the mouse results in extraordinarily high concentrations of $IgG_1$ in the serum. They showed that at least 10 mg /ml of this immunoglobulin isotype is specific for antigens of parasite origin. Sher, Smithers, Mackenzie and Broomfield, (1977) demonstrated also that a degree of protection could be transferred by a fraction of immune serum enriched in $IgG_1$. However the fractions used were contaminated to varying degrees with $IgG_{2a}$ (5-25%).

A striking phenomenon of this and many other helminth infections is the consistent eosinophilia which accompanies them. This response may be correlated with the appearance of specific IgE antibody in helminth infections and hyper-sensitivity states, although the two responses are probably not directly related.

Eosinophils are believed to arise in the bone marrow from a stem cell, the myeloblast. It is impossible to say whether or not this precursor cell also gives rise to the neutrophil and basophil but there is some evidence to suggest that the precursors of the latter two cells are distinct (Reviewed Zucker - Franklin, 1974; Hudson, 1968).

The eosinophil response seems to be regulated by the T lymphocyte (Basten, Boyer and Beeson, 1970; Basten and

Beeson, 1970), and appears to be controlled at the stem cell level by soluble factors released from specifically sensitised T lymphocytes upon contact with specific antigen (Colley,1973; Miller, Colley and McGarry, 1976). The study of eosinophilia in nude mice by Mitchell, et al., (1977); Hsu et al., (1976) and Ruitenberg et al., (1977) supports the idea that the eosinophil response is a T cell dependent phenomenon.

Despite the striking and consistent nature of the relationship between helminth infection and eosinophilia it has been quite difficult to attribute a functional role to the eosinophil. Recently however, the functions of preferential phagocytosis of immune complexes, moderation of anaphylactic hypersensitivity reactions, (Coombs Type 1, Coombs and Gell, 1975) and the mediation of damage to parasites have been ascribed to the eosinophil, (Reviewed, Butterworth, 1977). Eosinophils will phagocytose bacteria and other particles, but usually with less efficiency than neutrophils, (Cline, Hanifin and Lehrer, 1968). However the phagocytosis of immune complexes is particularly efficient. Phagocytosis is generally followed by degranulation and sometimes discharge of the granule contents outside the cell (Cline et al., 1968; Takenaka et al., 1977). Circulating immune complexes have been described in many disease states and have sometimes been implicated as playing a part in pathogenesis of these conditions. The phagocytosis of these complexes may help moderate the effects of some of these disease states.

Another postulated role for eosinophils is the moderation of anaphylactic reactions. This seems to be done in several

stages, by preventing mediator release, (Hubscher, 1975) by inactivating mediators such as histamine and slow reactive substance of anaphylaxis (SRS-A) once they have been released, (Zieger, Yurdin and Colten, 1976; Wasserman, Goetzl and Austen, 1975) and by modifying mediator replenishment after the reactions have occurred, (Jones and Kay, 1976). It is possible that because of the high IgE levels induced by helminth infections and in atopic individuals anaphylactic reactions become more frequent and eosinophils may play a role in moderating these reactions.

The eosinophil has also been implicated in immunity to this infection from the results of in vivo experiments. Mahmoud, Warren and Peters, (1975) found that immunity of mice to reinfection could be ablated by treatment of immune animals with a specific anti-eosinophil serum. They could not detect any difference between controls and animals treated with anti-neutrophil, anti-macrophage or anti-lymphocyte serum.

In an attempt to clarify which cells were involved, many studies investigating the killing of schistosomula in vitro, in the presence of various cell types, antibody and complement have been carried out. The in vitro experiments of Butterworth et al., (1977) showed that human eosinophils could kill schistosomulae in the presence of specific antibody. Antibody dependent damage to schistosomula by eosinophils from immune rats, (Mackenzie et al., 1977) and monkeys, (Li Hsu et al., 1977) has also been demonstrated. Some eosinophils may have an Fc receptor which enables them to adhere to schistosomulae coated with IgG antibody (Butterworth

et al., 1976). There is further evidence that rat eosinophils may have a C3 receptor, since the ability of these cells to bind to schistosomulae was lost if the serum was heated, (Ramahlo-Pinto, McLaren and Smithers, 1978). Serum from immune animals may damage schistosomulae in the presence of complement. This is usually determined by the release of $^{51}Cr$. from isotopically labelled larvae, the exclusion of methylene blue, or by observation of the morphological changes in the structure of the larvae (Clegg and Smithers, 1972; Li Hsu et al., 1977). However, the role that this mechanism might play in vivo in determining immunity is obscure.

In contrast to the results obtained with anti-eosinophil serum in vitro, studies by a number of other workers have implicated neutrophils, macrophages and lymphocytes as cells also involved in antibody dependent killing of schistosomulae (Li Hsu et al., 1977; Perez and Smithers, 1977). Rat macrophages have also been shown to be cytotoxic effector cells by Capron et al., (1977). These investigators showed that macrophages became activated following the ingestion of IgE-antigen complexes. These were then able to kill schistosomulae of S. mansoni in the presence of specific IgE antibody.

All these studies indicate that the invading schistosomulae may be killed by a number of cell types directed by specific antibody. The importance of one cell type as opposed to another may depend on where the schistosomulae are trapped and killed i.e. in the skin or the lungs.

## 3.4 Mechanisms of immunity to Trichinella spiralis

T. spiralis is a gastrointestinal nematode infecting man, ruminants, pigs and rodents. It is estimated that 4% of the population of the United States suffers from trichinosis. Infection occurs by ingesting meat containing encysted larvae. The acidity of the stomach releases the larvae from the cysts which then enter the intestine and after two moults they reach maturity. The females migrate to the mucosa to lay eggs. The larvae hatch, enter the circulation via the lymphatic system and after circulating through the heart and pulmonary vessels they eventually encyst in the skeletal muscles. The encysted larvae may remain alive,despite calcification of the cysts,for a number of years. After copulation the male adults die and are expelled but the gavid female adult worms may survive for several weeks, in the intestine, depending upon the species of the host, but are finally lost. Studies on immunity have been directed at the mechanisms involved in the expulsion of adult worms and those leading to a reduction in the number of muscle larvae. It is important to note that most of the pathologic effects of infection arise from the passage of larvae through the body and their encystment.

Infection of most animals by T. spiralis results in both an IgE response and an eosinophil response. The mechanism of eosinophilia has been studied in detail by Basten, Boyer and Beeson, (1970) and Basten and Beeson, (1970) and is worth considering in detail. They showed that in a natural infection in rats, eosinophilia develops on the 6th

day, at the time the female penetrates and deposits the new larvae in the intestinal mucosa. If the larvae were injected intraveneously eosinophilia developed more rapidly and could be correlated with an accute inflammatory response in the lungs. The appearance of eosinophils was not related to the appearance of specific antibody. If the rats were irradiated and thymectomised or depleted of lymphocytes by thoracic duct drainage there was a significant reduction in the eosinophil response. The eosinophil response in irradiated rats could only be restored by the injection of lymphocytes and bone marrow. If the rats were reconstituted with lymphocytes or thoracic duct lymph from immune animals and normal bone marrow, an elevated eosinophil response occurred. This response could be elicited by a soluble factor released from sensitised lymphocytes.

Thus in this case eosinophilia was shown to be an immune response dependent upon the presence of T lymphocytes. The response was mediated by soluble factors, probably released from sensitised T lymphocytes upon contact with specific antigen.

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

The indication that antibody was involved in expulsion of adult worms was supported by the transfer of immunity to rats by a purified B lymphocyte population. This immunity caused enhanced loss of adult worms and hence greatly reduced the numbers of larvae encysting in the muscles (Despommier et al., 1977). Since immunity could also be transferred with a sensitised population of thymus derived lymphocytes the antibody involved probably results from the interaction of these lymphocytes and host B lymphocytes (T dependent antibody).

The proposal has been put forward that expulsion of adult worms was due to a specific D.T.H. reaction caused by contact between antigen and sensitised T cells. This resulted in a non-specific inflammatory reaction which caused tissue damage and created an unfavourable environment for the adult worms (Larsh and Race, 1975). These investigators have shown that there is a good correlation between adult worm expulsion and intestinal inflammation. However firm evidence that a cellular hypersensitivity response is involved is lacking. Their experiments in which mice were treated with anti-thymocyte serum would inhibit also T lymphocyte dependent antibody responses as well as cellular hypersensitivity reactions. Further, they have never looked at the effects of anti-thymocyte serum on the antibody response to a heterologous antigen and hence may not be justified in drawing conclusions concerning the role of cellular hypersensitivity in immunity to _T. spiralis_.

Mice may be protected against *T. spiralis* infection by the intraperitoneal injection of an extract of muscle larvae in complete Freunds adjuvant. Immunisation caused a maximum reduction of 98% in the numbers of larvae in the muscles and enhanced expulsion of adult worms. (Despommier, Campbell and Blair, 1977). The mechanism involved in the reduction in the number of larvae is not known, although there is some evidence that eosinophils may be involved. Grove, Mahmoud and Warren, (1977) showed that mice immune to *T. spiralis*, which had been treated with an anti-eosinophil serum had 50% higher numbers of larvae in the skeletal muscles than untreated controls.

Thus the immune mechanism(s) which expell *T. spiralis*, differ again from the other 2 helminths discussed. Immunity seems to be predominantly dependent upon T. dependent antibody, and may be induced by immunisation with a larval extract. Eosinophils are probably involved in killing some larvae in immune animals, but further study is needed to determine what other mechanisms are involved.

3.5 Helminth infections in nude and T. cell depleted mice

The manifestations of immunity mediated by thymus derived lymphocytes (T. cells) cover a wide range of responses. T. cell mediated immunity is usually manifested in the delayed type hypersensitivity reaction (D.T.H.). This reaction involves the slow accumulation of leukocytes at the site of injection of antigen into a sensitised animal. There are numerous *in vitro* correlates of D.T.H. such as macrophage

migration inhibition and blastogenesis, many of which are dependent upon the release of soluble factors, (lymphokines) following contact between sensitised T. lymphocytes and the specific antigen. Transfer of D.T.H. reactions from a sensitised to a non-sensitised animal may be carried out using a pure T. lymphocyte population. Positive *in vivo* or *in vitro* cell mediated reactions are an indication of T. cell recognition of antigen and involvement in immune responses (Reviewed, Crowle, 1975).

In the study of immunity to nematodes, protection has often been transferred from an immune animal to an uninfected animal with mesenteric lymph node or spleen cells. It has been implied from this that cell mediated immune mechanisms are probably involved. However since the lymphocytes transferred contained both sensitised T. and B. cells an adoptive antibody response would be elicited as well as T. cell dependent cellular responses.

Recognition of parasite antigens by T. cells has been demonstrated in a number of helminth infections. More recently studies of infections in congenitally athymic (nude) mice have provided valuable information concerning the role of T. cell dependent immune responses in the development of resistance to these parasites.

In nude mice, studies have indicated that expulsion of some nematodes and cestodes from the intestine is highly dependent upon T. cells, for example, adult worms of *N. brasiliensis* were not lost from the intestine of nude

mice (Jacobson and Reed, 1974; Mitchell et al., 1976 ) whereas they were from the intestine of intact animals. This is in agreement with the findings of Ogilvie et al., (1977) in rats, discussed earlier.

Investigating T. spiralis infection in mice, Ljungstrom and Ruitenberg, (1976) showed that intact mice produced higher levels of antibody against the parasite than did T. cell-depleted mice. Ruitenberg et al., (1977) went on to show that nude mice did not expel adult T. spiralis and that infected nude mice had 4-5 times higher number of muscle larvae than the intact infected mice. T. cell depleted mice also lost the ability to rid themselves of Trichuris muris and this capacity was restored by the injection of a high dose of thymocytes again emphasising the dependence of expulsion on the presence of T. cells (Wakelin and Selby, 1974). Nude mice also failed to reject the cestode, Hymenolepis nana from the intestine (Isaak, Jacobson and Reed, 1977).

Studies of infections in nude mice have shown that the lack of T-cell dependent responses may affect the susceptibility of the host to other parasites in a number of ways besides their inability to expell helminths. The cestode, Mesocestoides corti multiplies in the liver of infected intact mice for many months. However in the nude mouse the multiplication rate is increased and the parasite quickly overwhelms the host (Mitchell et al., 1977). This suggested that M. corti larvae are capable of inducing a T-cell dependent immune response which results in a reduced proliferation rate but

never elimination of the parasite. Mitchell, Goding and Rickard, (1977) have also shown that nude mice were more susceptible to a primary infection with Taenia Taeniaeformis than were intact mice. This indicates that a T-cell dependent response may prevent cysts from establishing in the livers of infected mice.

Some of the eggs laid by the trematode, S. mansoni in infected mice become trapped in the tissue and result in the formation of granulomatous lesions. In nude mice these granulomata are significantly reduced in size, intensity of cellular infiltration and collagen deposition (Hsu et al., 1976; Bryam and Von Lichtenburg, 1977; Phillips et al., 1977). Bryam and Von Lichtenburg, (1977) suggested that this reduced granuloma forming ability in nude mice resulted in their failure to sequester toxic egg products resulting in extensive liver cell damage.

These studies emphasise the importance of T cell dependent functions in the development of resistence to these parasites.

## 4. Nematospiroides dubius infection in mice

### 4.1 Life cycle

Nematospiroides dubius was first described by Baylis in 1926. The life cycle has been studied since by many workers including Spurlock, (1943); Ehrenford, (1954); Fahmy, (1956) and Baker, (1954 and 1955). The most recent work on the life cycle has been done by Bryant, (1973). She found that the eggs in the faeces of infected animals contained fully

developed larvae within 24 hours.

Thirty six to thirty seven hours after laying, the eggs hatched to give rise to 1st stage larvae, which moulted for the first time 28-29 hours later. At approximately 4.5 days of age, the larvae underwent a partial moult to become ensheathed, non-feeding but active, infective third stage larvae ($L_3$).

These infective third stage larvae, when ingested by the host, exsheathed in the stomach within several minutes. By 24 hours most had penetrated the intestinal mucosa and by 2 days post infection differentiation to male and female worms was apparent. The larvae moulted once more, 90-96 hours after infection to become fourth stage larvae. These larvae then encysted in the muscularis mucosa. This cyst was of parasite origin and not host derived. A possible further moult took place at 144-166 hours post infection and by 191 hours the young worms had entered the lumen and penetrated deep into the Crypts of Lieberkuhn (Baker, 1954). The first eggs were laid approximately two days later.

4.2 Pathology of infection

A heavy infection caused emaciation of the mice, roughness of the coat and a reddish brown diarrhoea, probably due to intestinal haemorrhage (Spurlock, 1943). This haemorrhage was caused by the larvae penetrating the wall of the small intestine which may have resulted in the release of necrotising substances (Baker, 1955; Liu, 1965a). As a consequence of this, the small intestine became inflamed,

enlarged and fragile. Blood clots were seen in the lumen and anaemia and subsequent splenomegally resulted (Baker, 1955). Peristalsis seemed to be reduced and the enteric contents were frothy (Baker, 1954).

Four hours following ingestion, penetration of the epithelium commenced and after 12-24 hours the basement membrane had ruptured and necrosis in the muscularis mucosa was noticed (Liu, 1965a). Neutrophil infiltration was seen in the submucosa (Liu, 1965a) and in the muscularis mucosa (Panter, 1969a). After 4 days, the larvae began to encyst in the muscularis mucosa and macrophage infiltration occurred (Baker, 1954). The cysts were large, white and opaque by day 6 (Liu, 1965a) when the larvae were beginning to emerge from the cyst as juvenile worms. The vacated cysts became filled with cells, mainly neutrophils and macrophages and a few eosinophils (Baker, 1954; Liu, 1965a; Panter, 1969a). The adults appeared in the lumen, deeply entwined in the villi with their anterior end deep in the Crypts of Lieberkuhn. There was marked atrophy of the mucosa (Baker, 1954) and Panter, (1969a) claimed the worms fed on host tissue. Baker, (1954) noted also, an increase in goblet cells and an increase in secretory and exudative products. By day 13-15 the granulomata began to regress and by day 20-21 there were barely any remaining (Liu, 1965a; Baker, 1954).

Upon subsequent infections after 2 days there was marked necrosis of the mucosa and a cellular infiltration by polymorphonuclear cells, plasma cells, macrophages and eosinophils (Liu, 1965b). The cysts were surrounded by an

eosinophilic halo and the emergence of juvenile worms was greatly delayed, (Jones and Rubin, 1974). The lesion then developed a neorotic centre and became a firm yellow or white granuloma. By 49 days fibroblasts were present and by day 73 post infection, regression had commenced. By day 90 few granulomata remained.

4.3 Changes in cell levels induced by infection

Baker, (1962) demonstrated that over the course of 25 days following one infection there was a five-fold increase in the total blood leucocyte number. The increase peaked at day 5-8 corresponding with the period of larval emergence from the cysts. He showed a relative increase in lymphocyte and neutrophil numbers and demonstrated that the leukocytosis was not due to bacterial infection or anaemia. Cypess, (1972) found that in reinfected animals there was a reversal of the blood neutrophil/lymphocyte ratio which he considered to be due to inflammation about the time of larval killing. He demonstrated also a relative blood eosinophilia 16 days following the 2nd infection to a level of 15% and noted that the leukocyte level rose within 4 days after challenge in immunised animals which is suggestive of an anamnestic response.

4.4 Changes in immunoglobulin levels during infection

Crandall, Crandall and Franco, (1974) found that there was a small rise in serum IgM and an extraordinarily large increase in serum $IgG_1$ concentration following oral and subcutaneous immunisation. There were increases in $IgG_{2a}$

and IgA, but to a lesser extent. Upon studying intestinal responses during secondary infections it was shown by immunofluorescence that $IgG_1$ plasma cells were the most common infiltrating cell. In intestinal washes, IgA and $IgG_1$ were detected, but antibody was found only in the $IgG_1$ isotype. However, Cypess, Ebersole and Molinari, (1977) found antibody in the IgA class in intestinal washings but due to the technique of determination of immunoglobulin isotype levels before and after adsorption with antigen used, the significance of the results is doubtful. No IgM or $IgG_{2a}$ antibody was detected in the intestinal washes. Because of the intestinal haemorrhage which occurs during infection, it is not at all surprising to find $IgG_1$ in the lumen of the intestine and is therefore difficult to draw conclusions on the importance of intestinal antibody in immunity from these findings. The antibody levels were determined by immunofluorescence against sections of adult worms (Crandall et al., 1974) or by adsorption with adult antigen (Cypess et al., 1977). This makes these results difficult to interpret since Van Zandt, (1961), Panter, (1969a) and Bartlett and Ball, (1972), have demonstrated that immunity is elicited by and directed against the 3rd stage larvae.

Two important facts which do come out of the above studies are the presence of an enormous primary $IgG_1$ response and the demonstration of the secondary antibody response to multiple infections. Also evident was the lack of firm evidence for the presence of secretary IgA specific antibody during infection, despite intestinal tissue damage.

4.5 Development of immunity to N. dubius in mice

The first attempts to demonstrate that mice could develop immunity to N. dubius infection were carried out by Spurlock, (1943). He showed that one infection of 50 3rd stage larvae administered orally did not induce immunity to subsequent re-infection. The development of immunity was first demonstrated by Van Zandt, (1961) who showed that mice developed immunity after 3 stimulating infections. He also showed that penetration was required for the development of immunity and the adult worms themselves did not seem to be important in stimulating a protective response. This was confirmed by Panter, (1969a) and Bartlett and Ball, (1972) who showed that the life span of the adults was not reduced during repeated infections with 3rd stage larvae. They determined that the minimum dose required to produce immunity was 150 infectious 3rd stage larvae administered orally in 3 doses of 50 at 10 day intervals.

Liu, (1965b) studied the pathology of secondary infections and noted that granulomata formation was more severe in these animals. He concluded that the granulomata in the 2nd infection resulted from the larvae being killed by an immunity which developed as a result of the previous infection. This view of immunity was opposed by Panter, (1967, 1969b) who suggested immunity was due to immediate type hypersensitivity reactions. She demonstrated that multiple infected mice mounted active cutaneous anaphylactic (A.C.A.) responses to extracts of adult worms, and sera from these animals could be used to induce passive cutaneous

anaphylactic reactions (P.C.A.) with the adult worm antigen extract. She showed further, that when mice which had been previously sensitised to horse serum underwent simultaneous anaphylactic shock and oral challenge with infectious 3rd stage larvae, no penetration of the intestinal mucosa occurred and assumed that they had been expelled. However, immune mice do not develop anaphylaxis when given 3rd stage infectious larvae orally or systematically, and the P.C.A. and A.C.A. reactions were done with adult extracts despite the fact that it had been shown that the adults were not directly involved in immunity (Van Zandt, 1961; Bartlett and Ball, 1972; Chaicumpa, 1973). Indeed, attempts have been made to repeat this experiment by Crandall, Crandall and Franco, (1974) and Chaicumpa and Jenkin, (personal communication) but have been unsuccessful. It seems therefore unlikely that this is the mechanism of immunity.

Leuker, Rubin and Anderson, (1968) induced immunity in mice by giving one injection of 4,000 3rd stage larvae subcutaneously. They did not however, count cysts or adult worms which reached the intestine as a result of this immunisation and may be potentially important in the induction of resistance. This was confirmed by Rubin et al., (1971) and Cypess and Zidian, (1975) who showed that following subcutaneous vaccination with 2,000 3rd stage larvae approximately 1% developed into adults in the small intestine. However, no one has determined the number of larvae which encyst at the site of injection.

The notion of immediate hypersensitivity playing a role

in immunity was furthered by Jones and Rubin, (1974). They showed that in animals immunised orally the challenge dose was rapidly removed from the intestine whereas in subcutaneously immunised animals the challenge dose was removed slowly. In contrast to this the results of Nielson, Forrester and Thompson, (1973) indicate that the challenge dose is not lost from the intestine of immune mice. Larval parasite numbers in the intestine in both studies were determined by direct counts which are unreliable. It was also claimed that there were fewer granulomata in orally immunised mice but no direct counts to back up this claim were given. Jones, (1974) furthered the concept that there was a difference in mechanisms of resistance between orally immunised and subcutaneous immunised animals by claiming that orally immunised animals showed a greater P.C.A. reaction than subcutaneously immunised animals. This was determined by comparing mean diameter and intensity of the reactions, a very subjective and unreliable procedure. From this it was concluded that there was a higher production of reaginic antibody following oral immunisation than subcutaneous immunisation, hence implying that orally immunised mice have a greater potential than subcutaneously immunised mice for immediate hypersensitivity reactions causing expulsion of a challenge infection. After demonstrating that anti-thymocyte serum and anti-histamines abrogated immunity induced in subcutaneously immunised animals but not in orally immunised animals it was suggested that immunity in orally immunised animals was due to an I.T.H. mediated expulsion and that

immunity in subcutaneously immunised animals was dependent upon the action of sensitised lymphocytes and possibly on biogenec amine release.

However, Bartlett and Ball, (1974) have shown by macrophage migration inhibition studies that host T cells do recognise adult worm antigens although their earlier studies indicated that adult worm antigens are not involved in the stimulation of immunity. Despite this, the T cell depleted mice used by Bartlett and Ball, (1974) did not accumulate larger worm burdens than intact resistant mice so the role of T cells is still unclear, although the ability of Chaicumpa, (1973) to transfer protection to normal mice with thoracic duct lymphocytes from immune mice supports the concept of a role for T cells in immunity.

These results, together with the results of Chaicumpa, (1973) who showed that either spleen cells or peritoneal exudate cells from immune animals in combination with immune serum <u>in vitro</u> could reduce the infectivity of 3rd stage larvae, supports the idea that immunity may involve mechanisms other than I.T.H.

## 4.6 Susceptibility and development of resistance in inbred mice to <u>N. dubius</u>

Studies already described have shown that mice develop resistance to <u>N. dubius</u> infection following oral, subcutaneous or intravenous administration of larvae. The initial indication that there were differences in the development of immunity between inbred mouse strains resulted from reports by Rubin et al., (1971) that a number of inbred strains of

mice developed poor immunity to a single subcutaneous administration of live 3rd stage larvae. Again there is no indication of the number of larvae reaching the intestine as a result of this dose. Indeed both Leuker and Hepler, (1975) and Cypess and Zidian, (1975) showed that inbred mice could be effectively immunised by oral presentation of two or three doses of larvae.

Thus it seems apparent that different strains of mice may vary in their ability to develop protective immunity but the results so far are difficult to interpret because of the different doses and different modes of immunisation used.

4.7 Longevity of adult worms

There is very little information on the longevity of adult worms in the small intestine of mice, either following a primary infection or after multiple infections. Ehrenford, (1954) indicated that they had a potential lifespan of 32 weeks and the results of Liu, (1966) indicate that the 50% survival time of adult worms was 27 weeks in the $C_3H$ mouse and 20 weeks in the Webster mouse. However, Cypess et al., (1977) has described the gradual loss of adult worms from (A/He x C57L)$F_1$ mice, 65% of adult worms being lost in less than 3 weeks. A similar loss of adults has been described by Jenkins, (1977) from the jird, Meriones unguiculatus where almost all adult worms are lost by day 35 following a primary infection. He suggested that the adult worms were expelled as a result of an immunological reaction. It is worth noting that the jird developed full resistance to reinfection after only one exposure, with the female developing

better resistance than the male.

The longevity of adult worms in immune animals was investigated by Bartlett and Ball, (1972). They concluded that adult worms were not lost from animals during the development of protection. However, these mice could resist only 65% of a challenge dose of larvae. Cypess and Van Zandt, (1973) described the loss of adult worms in immune animals within 2 hours of administration of 3rd stage larvae orally and Cypess and Zidian, (1975) reported expulsion of *N. dubius* adult worms from Swiss white mice but not from BALB/c, C57Bl/6 or ICR/$CD_1$ mice. Unfortunately they did not give any parasite counts which makes their report difficult to interpret.

It is clear from the above studies that, there may be a slow loss of *N. dubius* adult worms following a primary infection in some strains. The results of Dobson and Owen, (1977) indicate that this may depend upon the strain of mice in which the parasite is passaged. Loss of adult worms from immune animals does not occur in male CFW mice (Bartlett and Ball, 1972) but may occur in other strains of mice. There is a need for this phenomenon to be investigated further since it is impossible to draw conclusions from much of the work due to the lack of data.

### 5. *N. dubius* infection as a model for human hookworm infection

Bartlett and Ball, (1972) proposed that *N. dubius* infection in mice may be a good model for human hookworm infection in man. Despite some marked differences in the

life cycle, there is a remarkable similarity in the migration of the larvae to the intestine by N. dubius following intravenous injection and by hookworm following the penetration of the skin. Another important similarity is the persistance of adult worms in the intestine despite continuous exposure to larvae.

Increased knowledge of the immune mechanisms which may prevent larvae from migrating through tissue and which are involved in the persistance of adult worms may help increase our understanding of the interactions between hookworms and their hosts.

# CHAPTER 2

## Materials and Methods

1. Mice

2. Maintenance of the Parasite *Nematospiroides dubius* (*Heligmosomoides polygyrus*)

3. Infection and Immunisation

4. Assay of resistance to infection with *N. dubius*

5. Collection of serum

6. Cell collection and culture

7. Cell counting - total and differential cell counts

8. Adherence of peritoneal exudate cells to third stage larvae

9. *In vitro* assay for the effect of peritoneal exudate cells on third stage larvae

10. Preparation of cell suspensions for injection

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12. Iodination of immunoglobulins

13. Immunoglobulin determinations

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15. Preparation of IgG

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    15.2 Protein A - Sepharose 4B

    15.3 Sephadex G-200 chromatography

16. Statistics.

1. Mice

The mice most frequently used in this study were LACA mice. They were raised under specific pathogen free, (S.P.F.) conditions in a closed, outbred colony at the University of Adelaide Central Animal House. They were transferred to conventional conditions at the age of 6 weeks and used 2-4 weeks later.

The inbred mice were obtained from the Walter and Eliza Hall Institute of Medical Research S.P.F. animal facility. The strains of mice used were CBA/CaH Wehi, C3H/Hej Gif Wehi, BALB/c An Bradley Wehi, SJL/J Wehi, C57Bl/6J Wehi, A/J JAX, and DBA/2. The hypothymic mice used were BALB/c nu/nu and their intact normal littermates. All mice were maintained under conventional conditions.

2. Maintenance of the parasite *Nematospiroides dubius* (*Heligmosomoides polygyrus*)

Infective third stage larvae ($L_3$) of *Nematospiroides dubius* were supplied by Dr. R.I. Sommerville of the Zoology Department, University of Adelaide. Female LACA mice received an oral administration of 200-400 $L_3$. These infected mice were used as a source of eggs. Fresh mice were infected on average, every 3-4 months.

Wire mesh grids were placed in the bottom of cages over moist filter paper to collect the faecal pellets, and mixed with distilled water. The slurry was passed through a 500μ

sieve. After centrifuging the filtrate at 1,5000 g for 15 minutes, the supernatant was discarded and the pellet resuspended in distilled water. The suspended sediment was then streaked onto moist Whatman filter paper lining a large petri dish or an 8 × 8 cm. glass tray. These were then left at room temperature under high humidity.

After seven days of culture the larvae were collected by rinsing the filter paper in the dishes with distilled water. This was repeated on the eighth day of culture. The larvae were washed and re-suspended in distilled water and maintained at $4^{o}$ until used. The third stage larvae could be maintained for periods of greater than one month and remain fully viable and infective.

The concentration of larvae in the suspension was determined by counting the number in an 0.1 ml aliquot placed on a perspex grid, covered with a coverslip, under a dissecting microscope.

3. Infection and Immunisation

Third stage larvae of N. dubius were administered orally to mice by introducing a given number in 0.2 ml of physiological saline into the lower part of the oesophagus using a blunt 19 guage needle connected to a tuberculin syringe. Mice were immunised routinely by administration of 2 or 3 doses of $L_3$, either orally or intravenously at 12-14 day intervals. Mice were also immunised by the intraperitoneal injection of 3 doses of 200 $L_3$, 14 days apart or by the subcutaneous injection of 2 doses of 2,300 $L_3$ beneath the skin at the back of the neck, 21 days apart.

4. Assay of resistance to infection with *N. dubius*

The level of resistance was determined by comparing the number of encysted larvae in immunised mice with the number in naive mice following an oral challenge with $L_3$. The same batch of $L_3$ was used within each experiment. Adult worms and granulomata which arose from the previous immunising infections were also counted in some experiments. The granulomata resulted from the cellular inflammation around the vacated cysts and particularly around killed larvae from the previous infections. The number and persistence of adult worms and granulomata provided a measure of the host's immune response. In some experiments the adult worms arising from the immunising infections were cleared by the oral administration of an anti-helminthic, Pyrantel pamoate, (Pfizer) at a dose of 12 mg /Kg body weight.

Cysts were counted on the 5th day after challenge. The small intestine was removed completely and pressed between two pieces of transparent perspex. The whole of the exposed intestine was scanned under a dissecting microscope and the cysts counted. Granulomata could also be counted at the same time in immune animals. To count the adult worms the small intestine was split longitudinally and placed in either, a petri dish containing saline which was heated under a lamp, or in a Baermann jar which was placed in a $37^{\circ}$ waterbath. The worms became active and moved away from the intestine and could be removed with a pasteur pipette and counted.

5. Collection of serum

Mice were bled from the retro-orbital plexus and the blood allowed to clot at room temperature. After incubation overnight at 4$^{o}$ the serum was collected and stored at -20$^{o}$ until required.

6. Cell collection and culture

Peritoneal exudate cells were used most commonly throughout this study and were collected in the following manner. Mice were killed and the skin over the abdomen deflected. Four ml of ice cold 199 medium (Commonwealth Serum Laboratories) which contained 200 units/ml penicillin, 200 mg /ml Streptomycin sulphate, 5 I.U./ml of heparin and buffered to pH 7.4 with 10 mM Hepes, (Calbiochem) was then injected into the peritoneal cavity. The abdomen was massaged gently and the fluid withdrawn with a 19 guage needle mounted onto a 5 ml syringe. The cells were then treated in one of 3 ways.

Peritoneal exudate cell suspensions were prepared by squirting the cells into a siliconised tube on ice. The cells were washed at least twice in heparin free 199 medium supplemented with 10% heat inactivated foetal calf serum, (199 + F.C.S.) by centrifuging at 500 g for 5 minutes. A sample of the cells was then diluted in a white cell counting fluid (section 7) and counted in a haemocytometer.

Monolayers of peritoneal exudate cells were prepared by gently squirting the cell suspension into Leighton tubes. After 30 minutes incubation at 37$^{o}$, the medium was replaced with 199 + F.C.S. The monolayers were used after a further

15 minutes incubation.

To prepare non-adherent cells the peritoneal exudate cells were allowed to settle onto plastic petri dishes for 30 minutes at 37°. The medium containing the non-adherent cells was removed after which the plates were washed in 2 ml of ice cold 199 + F.C.S. Both the non-adherent cells in the original medium and the cells in the wash were pooled, washed by spinning, resuspended and counted as described above.

7. Cell counting - total and differential cell counts

Leukocytes were collected by diluting a known volume of blood in a white cell counting fluid composed of 0.1% gentian violet in 10% v/v acetic acid/distilled water. A haemocytometer was filled with an aliquot of this and the leukocytes counted.

Peritoneal exudate cells were collected by injecting into the peritoneal cavity, 2 ml of ice cold isotonic phosphate buffered saline pH 7.4 (P.B.S.). One ml of fluid was withdrawn and diluted in white cell counting fluid. The number of cells was enumerated as above.

In order to carry out differential cell counts smears of blood and peritoneal fluid were made. They were stained with Wrights Blood Stain, (Difco) and at least 100 cells from each slide counted. One slide was made from each animal and the counts are the mean of 5 animals chosen at random from a pool of infected mice. Eosinophils could be identified on stained smears by virtue of their nuclear

shape and could be distinguished from neutrophils by the presence of bright red granules in the cytoplasm. Eosinophils could also be counted by diluting blood or peritoneal exudate cells in 10% acetone/distilled water (v/v) containing 0.1% (w/v) eosin yellow, (Gurr, Searle Diagnostic). Under these conditions the granules of the eosinophils stained bright red. These counts correlated well with the counts from smears.

8. Adherence of peritoneal exudate cells to third stage larvae

To study the adherence of peritoneal exudate cells to third stage larvae the following procedure was used. Sera were diluted twofold in 100µℓ of Veronal Buffered Saline (V.B.S.). This is an isotonic buffer containing 0.3 M. NaCl, 5mM. sodium barbitone, 0.15 mM. $Ca^{++}$ and 0.5 mM. $Mg^{++}$. The buffer sometimes contained ethylene-diaminetetra-acetic acid (E.D.T.A.), 0.01 M. or ethylene-glycol-bis (B-amino-ethyl ether) N, N'-tetra-acetic acid (E.G.T.A.), 0.01 M., as indicated in the text. To these tubes 100 $L_3$ in 100µℓ of the buffer used to dilute the serum, were added. The contents of the tubes were mixed well and incubated at 37° for 90 minutes with gentle agitation. This mixture was then diluted with 5 ml of isotonic saline and spun at 500 g for 5 minutes. The supernatant was withdrawn leaving the pelleted $L_3$ in approximately 200 µℓ. To this, 250 µℓ of a washed mouse peritoneal exudate cell suspension, collected as described

previously, containing $5 \times 10^5$ cells in 199 medium supplemented with 10% heat inactivated foetal calf serum, were added. The contents of the tubes were mixed and left to stand for 2-3 hours at room temperature after which the larvae were scored for adherence of cells in the following manner.

The larvae were removed from the tube and examined under a microscope at 100× magnification. This enabled the approximate number of cells adhering to each larvae to be counted and they were grouped as larvae with 0 cells, 1-10 cells and greater than 10 cells. Usually 30 larvae from each tube were counted. The end point could be easily distinguished and the titre of serum was taken as the highest dilution where $> 50\%$ of the $L_3$ scored had $> 10$ cells adhering to the cuticle.

9. *In vitro* assay for the effect of peritoneal exudate cells on third stage larvae

This assay was used to demonstrate the ability of various cell populations to damage $L_3$ *in vitro*. The basic assay described here was used in all *in vitro* experiments. Any variations from this method are described in the text.

The assay was carried out in sterile conical siliconised 12 ml glass tubes. The larvae used were pretreated in 0.02% sodium hypochlorite (NaOCl) for approximately 25 minutes at room temperature. This killed any bacteria and fungi which may have been on the surface of the larvae and also caused them to exsheath. The treatment did not reduce the viability of the larvae. After NaOCl treatment

the larvae were washed once in 199 medium supplemented with 10% heat inactivated foetal calf serum and finally suspended in the above medium at a concentration of between 2,000 - 3,000/ml Each tube contained 0.5 ml of larvae treated in the above manner. To these, 0.5 ml of heat inactivated serum was added, usually serum from immune mice or normal mice. Finally mouse peritoneal exudate cells, prepared as described previously, were added in 0.5 ml of the above medium. The number of cells used varied but was usually approximately $10^7$ per tube. The tubes were then incubated at $37^{o}$ for 20 hours with gentle agitation. At the end of the incubation period 0.2 ml samples of the contents of the tubes were fed to mice. Six 0.2 ml samples were taken from each tube and one fed to each of six mice. The number of cysts in each mouse was counted 5 days later. The results are expressed as the mean number of cysts in the 6 mice. By comparison of the larval infectivity between experimental and control tubes, which usually contained serum but no cells, the effect of cells on the larvae could be determined.

10. Preparation of cell suspensions for injection

Thymus and/or mesenteric lymph nodes were removed from BALB/c nu/+ or +/+ mice for the preparation of cell suspensions for injection into BALB/c nu/nu mice. The organs were disrupted through a 50 G stainless steel screen in Eisen's balanced salt solution (E.B.S.S.). The preparations were washed twice by centrifuging at 250 g for 7 minutes at $4^{o}$ in 10 ml of the above media. After washing the cells

were resuspended in E.B.S.S. and injected intraperitoneally into mice.

11. Collection of bone marrow cells

Bone marrow cells were collected by gently squirting 2 ml of cold 199 medium supplemented with 10% heat inactivated foetal calf serum (199 + F.C.S.) through the femurs of mice. The cells were pooled and separated by gentle pipetting, centrifuged at 500 g for 5 minutes and resuspended in 199 + F.C.S. They could then be stained and counted.

12. Iodination of immunoglobulins

The proteins, (50-100 μg) were labelled with carrier free $^{125}$I (IMS - 30, The Radiochemical Centre, Amersham) using the chloramine - T method of Greenwood, Hunter and Glover, (1973). The following procedure was used.

1. Add 1-2 μℓ of $^{125}$I (100 millicuries/ml) to the protein in 50 μℓ of phosphate buffered saline (P.B.S.) or 0.1 M. Tris-HCl pH 8.0, on ice.
2. Add 50 μℓ of chilled chloramine - T (20 μg/ml) in P.B.S., mix well and leave on ice for 90 minutes.
3. Following this procedure add 50 μℓ of sodium metabisulphite (1/400 dilution of a 6.5 mg/ml solution in P.B.S.), mix well and leave at room temperature for 5 minutes.
4. Add 25 μℓ of 0.1 M. potassium iodide and mix well.
5. Immediately, dilute the reaction mixture with 1.5 ml of 0.1 M. Tris-HCl pH 8.0 containing 0.1 M. NaCl, 0.1% sodium azide, 2% bovine serum albumin and 1 mM. ethylenediamine-tetra-acetic acid.

6. Dialyse against several changes of the above diluent or pass down a Sephandex G-25 column to remove unreacted iodine.

13. Immunoglobulin determinations

13.1 Radioimmunoassay

A radioimmunoassay, (R.I.A.),(Herzenberg, Warner and Herzenberg, 1965) was used most commonly to assay serum samples for their immunoglobulin isotype content. The precipitation of a known labelled mouse immunoglobulin isotype (usually 5 ng ) was carried out by mixing the mouse immunoglobulin with twofold dilutions of a goat antiserum specific for one particular isotype. A curve of precipitation versus antiserum dilution was thus obtained. Serum samples containing unknown amounts of a particular isotype were tested for their ability to inhibit the above reaction. By comparison with the degree of inhibition produced by a known amount of the specific isotype, the amount of that immunoglobulin class present in the serum sample could be determined.

The procedure for determining, for example, the amount of $IgG_1$ in a serum sample was as follows. In order to determine the concentration of specific anti-$IgG_1$ to use the antiserum must first be titrated against $^{125}I$-$IgG_1$.

1. Serially dilute 100 $\mu\ell$ of anti-$IgG_1$ in 100 $\mu\ell$ of 0.1 M. Tris-HCl, pH 8.0 containing 0.1 M. NaCl, 0.1% sodium azide and 10% normal rabbit serum (serum diluent).
2. Add 50 $\mu\ell$ of $^{125}I$-$IgG_1$ (5ng, 5-10,000 cpm) to each tube.

3. Mix well and incubate at $37^{o}$ for 3 hours, then $4^{o}$ overnight.

4. Spin the tubes for 90 minutes at 12,000 g and assay the radioactivity in 110 μℓ of the supernatant.

5. Dilute the anti-$IgG_1$ to a concentration twice that which precipitated 80-90% of the labelled $IgG_1$.

The amount of $IgG_1$ (inhibitor) in a serum sample was then determined as follows.

1. Serially dilute 50 μℓ of inhibitor (serum sample) in 50 μℓ of serum diluent.

2. Add 50 μℓ of pre-diluted, specific anti-$IgG_1$ to each tube.

3. Then add 50 μℓ of $^{125}I$-$IgG_1$ to each tube.

4. Mix the contents well and incubate for 3 hours at $37^{o}$ then $4^{o}$ overnight.

5. Spin the tubes at 12,000 g for 90 minutes and assay the radioactivity in 110 μℓ of the supernatant.

A known standard amount of $IgG_1$ (inhibitor) was also diluted at the same time so that the amount of $IgG_1$ in the serum sample could be determined. $IgG_{2a}$, $IgG_{2b}$, IgM and IgA were also determined in a similar manner using the respective monospecific antisera.

13.2 Radial immunodiffusion

Radial immunodiffusion was carried out in agarose gels (1% in tris-hydroxymethyl aminoethane-veronal buffered saline pH 8.6) containing monospecific antibody directed against mouse immunoglobulin isotypes. This was essentially as described by Fahey and McKelvey, (1965) and Mancini,

Carbonara and Heremans, (1965). The plates were washed by gently pressing between glass and rinsing (3×) in saline before being dried and stained with Coomassie Blue.

13.3 Antisera

Goat antibodies directed against mouse immunoglobulin heavy chains and standard mouse reference sera obtained from Meloy Laboratories (Springfield, Va.) were used throughout.

14. Determination of cell bound immunoglobulin

Peritoneal exudate cells were collected as described previously and washed 5 times by centrifuging at 500 g for 5 minutes in 199 medium supplemented with 10% foetal calf serum. The cells were counted (section 7) and their viability determined by trypan blue exclusion. A known aliquot was transferred to a small weighed polyallomer tube which was spun at 500 g for 5 minutes and the supernatant carefully removed. The tube plus cell pellet was reweighed and from the net weight (assuming a density of 1 gm/ml ) the volume of cells could be determined. The cells were then suspended in 199 medium containing 0.1% sodium azide to make a 50% v/v suspension.

Surface immunoglobulin was assayed by a R.I.A. using the cell surface immunoglobulin as inhibitor. The procedure was as follows.

1. Serially dilute 40 µℓ of the cell suspension with 40µl of 199 medium containing 10% heat inactivated foetal calf serum, and 0.1% sodium azide on ice. The first tube contains 40µl of undiluted cells.

2. Add 40 μℓ of antiserum specific for the heavy chains of one immunoglobulin isotype (concentration determined as described previously, section 12.1) in isotonic medium and mix well.

3. Incubate the tubes at 4° for 12-24 hours, mixing occasionally.

4. Spin the tubes at 2,000 g for 5 minutes.

5. Add 50 μℓ of the supernatant to tubes containing 50 μℓ (∿ 5 ng of $^{125}$I-mouse immunoglobulin isotype in serum diluent (section 12.1).

6. Incubate the tubes for 3 hours at 37° then at 4° overnight.

7. Spin the tubes at 12,000 g for 90 minutes.

8. Remove 60 μℓ of the supernatant and assay for radioactivity.

The amount of immunoglobulin on the cell surface was determined by comparison with the inhibition of precipitation by a reference mouse immunoglobulin isotype of known concentration.

## 15. Preparation of IgG

### 15.1 Octanoic acid fractionation

The modified method of Steinbuch and Andran, (1969) was used. To 100 ml of serum or plasma, 200 ml of acetate buffer (0.06 M. pH 4.0) were added. The pH was adjusted with sodium hydroxide to 4.8. With vigorous stirring, 6.8 grams of octanoic acid were added, dropwise. Stirring was continued for a further 30-60 minutes and the resulting

precipitate filtered through Whatman grade 1 filter paper. To each 100 ml of filtrate, 30 grams of solid ammonium sulphate was added. The mixture was stirred vigorously for a further 1-2 hours and finally centrifuged at 10,000 g for 30 minutes. The supernatant was removed and the deposit redissolved in 25 ml of phosphate buffered saline pH 7.4 (P.B.S.). After dialysis against P.B.S. the contents of the dialysis bag were spun at 5,000 g for 10 minutes and suitable aliquots kept at $-20^{o}$ until used. Analysis of the preparation on sodium dodecylsulphate (S.D.S.) polyacrylamide gels (kindly carried out by Mr. S. Schluter, Department of Microbiology and Immunology, University of Adelaide.), indicated the presence of only 2 bands which corresponded with the molecular weights of the heavy and light chains of IgG.

15.2 Protein A - Sepharose 4B

The procedure was carried out essentially as described by Ey, Prowse and Jenkin, (1978). The sera was diluted 1:2 with 0.14 M. sodium phosphate pH 8.0 and loaded onto a Protein A - Sepharose column, (Pharmacia) which had been equilibrated with 0.14 M. sodium phosphate pH 8.0. The column was washed with this buffer until no more protein was eluted as determined by O.D. 280 nm (Pool 1). Citrate buffer (0.1 M. pH 6.0) was then applied (30 ml) and fractions (4-5 ml) collected until no further protein came off the column (Pool 2). This process was repeated with 25-30 ml of 0.1 M. citrate pH 4.5 (Pool 3) and finally with 25 ml of 0.1 M. citrate pH 3.5 (Pool 4). The column was then equilibrated at pH 8.0. The fractionation was carried out

at 4° and the 4-5 ml fractions collected at a flow rate of 0.5 ml/min. Sufficient 1 M. Tris, pH 9.0 was added to the fractions to ensure neutralization before their removal from the cold. Four pools of serum proteins were obtained and were analysed by R.I.A. Pool 1 contained the bulk of the serum proteins, IgM, IgA and IgE; Pool 2 contained $IgG_1$; Pool 3, $IgG_{2a}$ and pool 4, $IgG_{2b}$.

The results of an elution of serum from immune mice and the analyses of the pools of eluted proteins are shown in Figure 2.1.

15.3 Sephadex G-200 chromatography

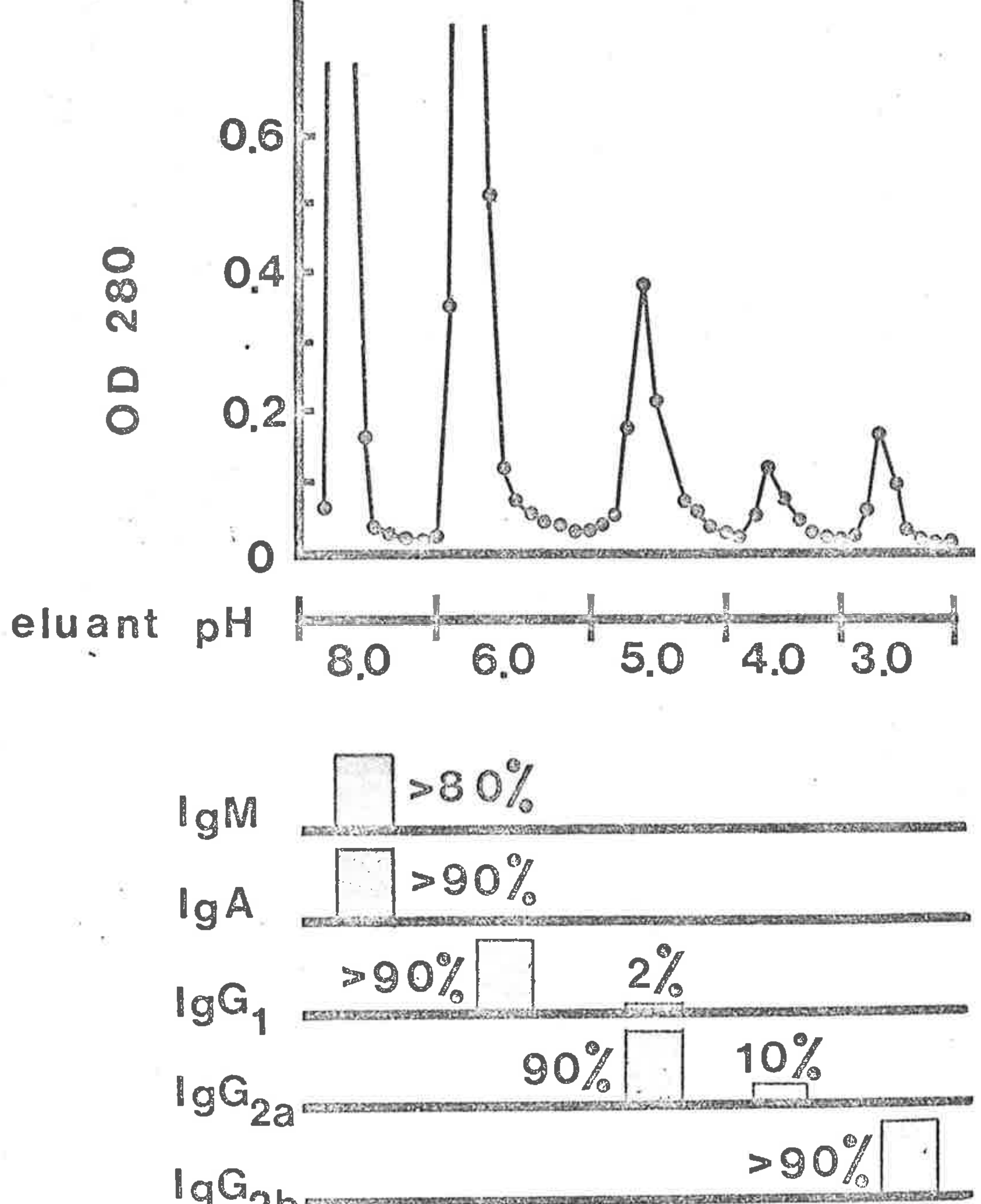
Serum was occassionally fractionated on Sephadex-G200. A 2 litre column (100 × 5.5 cm) was used at a flow rate of 36 ml/hour. The fraction size was 18 ml and all fractionation was carried out at 4°. The column was equilibrated in isotonic Tris buffer (tris (hydroxymethyl) aminomethane, 25 mM.; NaCl, 132.5 mM.; ethylenediaminetetra-acetic acid, 0.1 mM.; $NaN_3$, 8.3 mM.) pH 8.0.

## 16. Statistics

Two statistical tests were used to analyse data obtained in the following experimental chapters. If the data fitted a normal distribution as determined by plotting the rankits, (Sokal and Rohlf, 1969) and there was no significant difference between the variances as determined by an analysis of variance, a 'students' t test was used. Where the data was not normally distributed and/or the variance differed, the "U" test of Mann and Whitney was used. A probability of 0.05% or less was taken as being significantly different.

Figure 2.1

Elution of immunoglobulins from protein - A sepharose. 0.8 ml of serum from mice immune to reinfection by N. dubius was mixed with 2 ml of 0.14 M. phosphate buffer, pH 8.0, was applied and eluted as described. Elution at pH 4.5 can replace elution at pH 5.0 and 4.0 to elute the $IgG_{2a}$. The results are shown as block histograms depicting the proportion of immunoglobulin recovered as a percentage of that applied to the column.



OD 280
0.6
0.4
0.2
0
eluant pH
8.0
6.0
5.0
4.0
3.0
IgM
>80%
IgA
>90%
IgG1
>90%
2%
IgG2a
90%
10%
IgG2b
>90%

# CHAPTER 3

## The Development of Resistance in Different Strains of Mice to Infection with *N. dubius*

1. Introduction
2. Worm numbers following a primary infection in LACA mice
3. The development of resistance to *N. dubius* infections in LACA mice
4. The natural resistance of inbred mice to a primary infection and the development of aquired resistance
5. Development of resistance in inbred male and female mice
6. Survival of adult worms following a primary infection
7. Survival of adult worms in mice given repeated administrations of $L_3$
8. The loss of adult worms from mice which had either undergone a larval infection or which had received adult worms orally.
9. The development of resistance in (C3H/HeJ x CBA/H)$F_1$ and (CBA/H x C3H/HeJ)$F_1$ mice
10. Conclusions

1. Introduction

These initial investigations were carried out to study in more detail, the biology of the host-parasite relationship and in particular to choose a strain of mouse in which the adult worms parasited for a long period of time, thus mimicking hookworm infection in humans. Since large numbers of mice were likely to be used in future experiments it was desirable also to choose a strain in which males and females were equally susceptible to infection.

The first series of experiments determined the susceptibility of outbred male and female LACA mice following a single infection as measured by the number of cysts, adult worms and granulomata.

2. Worm Numbers Following a Primary Infection in LACA Mice

A group of 14 male and one of 15 female, 10 week old LACA mice received 200 $L_3$ orally on day 0. Five days later 5 mice of each sex were killed and the cysts counted. Ten days after infection the remaining mice were killed and the adult worms and granulomata counted. The results given in Table 3.1 show that LACA male and female mice were equally susceptible to a primary infection of _N. dubius_ as indicated by the number of cysts, adult worms and granulomata. The results show also that almost all the larvae which encyst go on to develop into adult worms.

Table 3.1

The incidence of intestinal cysts, granulomata and adult worms in male and female LACA mice following a single infection with *N. dubius* $L_3$

| Mice* | day 5 | | day 11 | | |
|---|---|---|---|---|---|
| | No. Mice | Cysts ± SE[+] | No. Mice | Adult worms ± SE | Granulomata ± SE |
| Male | 5 | 125 ± 5 | 9 | 129 ± 5 | 67 ± 10 |
| Female | 5 | 122 ± 8 | 10 | 119 ± 4 | 79 ± 10 |

* Mice aged 10 weeks on day 0.

+SE ≡ standard error

3. The Development of Resistance to *N. dubius* infections in LACA Mice.

In order to determine the ability of outbred LACA mice to develop resistance to infection, 10 male and 10 female LACA mice received 2 doses of 200 $L_3$ orally, 10 days apart.

Ten days after the final administration of larvae these mice, together with previously uninfected age and sex matched controls were challenged orally with 150 $L_3$. Five days later cysts were counted in the control group and cysts, adult worms and granulomata counted in the pre-infected group. In the latter group the adult worms and granulomata resulted from the two initial infections. This basic protocol is outlined in Figure 3.1 and was used in subsequent experiments.

From the data presented in Table 3.2 it may be seen that both male and female LACA mice developed good resistance to reinfection as indicated by the small number of cysts arising from the challenge infection compared with the number seen in the control animals. It was apparent however that they accumulated most of the adult worms arising from the immunising infections. These mice also had high numbers of granulomata, an indication of their ability to mount an inflammatory response. The female mice had slightly better granuloma forming ability than male mice as indicated by the significantly higher number of granulomata.

Since these outbred mice developed good resistance to infection it was of interest to study the development

Figure 3.1

Schedule used to immunise mice against *N. dubius* and to determine the level of resistance. Occasionally the first immunising infection was given on day -28 or -24 the second on day -14 or -12.

PRE-INFECTED MICE

| day | -20 | -10 | 0 | 5 |
|---|---|---|---|---|
| PRE-INFECTED MICE | 1st immunising infection | 2nd immunising infection | challenge infection | count cysts adult worms granulomata |
| NAIVE CONTROL MICE | | | challenge infection | count cysts |

NAIVE CONTROL MICE

Table 3.2

The development of resistance to N. dubius in male and female LACA mice*.

| Mice[¶] | One administration of $L_3$[†] | | Three administrations of $L_3$[‡] | | | |
|---|---|---|---|---|---|---|
| Sex | No. of Mice | No. of cysts (day 5) | No. of Mice | No. of cysts (day 5) | No. of adults (day 5) | No. of Granulomata (day 5) |
| M | 5 | 125 ± 5[§] | 10 | 1 ± 0.4 | 158 ± 14 | 141 ± 20 |
| F | 5 | 122 ± 8 | 10 | 1 ± 0.6 | 142 ± 19 | 223 ± 18[#] |

*Abbreviations: $L_3$ = third stage larvae of N. dubius, M = male, F = female.

† Mice received 150 $L_3$ orally on day 0.

‡ Mice received 200 $L_3$ orally on days -20 and -10 and 150 $L_3$ orally on day 0.

§ Arithmetic mean ± standard error

¶ Mice, 6 weeks old on day -20.

# "t" test, granulomata, male vs female $0.002 < P < 0.01$.

of resistance in inbred strains of mice. Several reports concerning the development of immunity to N. dubius indicated that mice of different strains may not develop similar levels of resistance, (Spurlock, 1943; Cypess and Zidian, 1975; Leuker and Heppler, 1975). Resistance to a challenge infection was assessed in these investigations, by counting adult worms in the luman. In addition, Liu, (1966) studied the natural resistance of inbred strains by following the mortality of the mice after a primary infection with third-stage larvae ($L_3$). Cypess and Zidian, (1975) concluded by counting adult worms that C57Bl/6 mice were protected against 50-60% of the challenge dose following oral or subcutaneous immunisation with $L_3$. In contrast, BALB/c mice were protected against 84% of the challenge dose of larvae following subcutaneous immunisation and 95-100% protection was achieved by 2 oral immunising infections with $L_3$. However, Leuker and Heppler, (1975) demonstrated that C57Bl/6 mice were protected against 95% of a challenge following oral immunisation and Behnke and Wakelin, (1977) showed that BALB/c mice could resist only 25% of a larval challenge infection following 11 oral administrations of 80 $L_3$. Adult worms were counted in all these investigations and thus it is impossible to determine from the data whether larvae administered as the challenge infection were killed at the cyst stage or whether adult worms arising from the challenge infection were expelled from the intestinal lumen.

In order to provide more detailed information on mouse strain variations in the development of resistance

to N. dubius infection, the consequences of infection have been examined in seven isogenic strains of mice. Resistance was evaluated by 3 criteria, namely (1) the ability to kill larvae encysting in the intestinal mucosa as reflected by a reduced number of cysts on day 5 after $L_3$ administration; (2) expulsion of adult worms from the lumen of the intestine as evidenced by a decreased adult worm burden with time after infection; and (3) formation of intestinal wall granulomata (i.e. "reactive foci") which develop as a consequence of the inflammatory response to larvae during their development in the intestinal mucosa.

4. The Natural Resistance of Inbred Mice to a Primary Infection and the Development of Aquired Resistance.

These experiments were designed to determine the resistance of naive mice (Group A), as well as mice given two immunising infections (Group B), to challenge with a standard dose of $L_3$. Group B mice of each strain received two oral doses of 200 $L_3$ 12 days apart. Ten days after the second dose, all mice, including uninfected age- and sex-matched controls (Group A above), were challenged with 150 $L_3$. These control animals served a dual purpose. Firstly, the natural resistance of each strain to a primary infection could be evaluated, and secondly, they provided a base line for the degree of resistance developed within each particular strain. Five days after challenge, all the immunised (i.e. previously exposed) mice and some of the controls were killed in order to count the number of

encysted larvae developing from the challenge. In the immunised groups of mice, the numbers of granulomata and adult worms persisting from the earlier infections were also determined. On day 19 post-challenge, the remaining control mice were killed and the adult worms arising from the primary infection counted. The results are presented on Table 3.3.

The differences in the number of cysts present in mice following a primary infection indicated that some strains may be somewhat more susceptible to this infection than others. The numbers of adult worms in some strains of mice (at day 19) were considerably less than the number of observed cysts (at day 5). This is contrary to previous observations in the LACA outbred strain where there was a good correlation between the number of cysts on day 5 and the number of adult worms on day 10 (Table 3.2; Chaicumpa et al., 1977). These differences between cyst and worm numbers can only be interpreted as a loss of adult worms. Compared with other strains, the C3H/HeJ and SJL/J mice lost the most adult worms by day 19 following a primary infection, and developed the most resistance upon immunisation as measured by all three criteria (see below).

The small number of cysts in the immunised mice compared to the naive control animals indicated that each strain was able to develop a substantial degree of protection against further challenge infection. However, some strains developed greater resistance than others. Comparing cyst numbers in female mice (Table 3.3), C3H/HeJ mice were

Table 3.3

Susceptibility of naive and immunised mice of different strains to infection by _N. dubius_

| Mice | | | Naive mice* | | Immunised (pre-infected) mice+ | | |
|---|---|---|---|---|---|---|---|
| Strain | Age (wk) (day 0) | Sex | No. cysts (day 5) | No. adults (day 19) | No. cysts | No. adults | No. granulomata** |
| CBA/H | 10 | M‡ | 102±3# | 92±2 | 35±8 | 312±12 | 42±7 |
| A/J | 10 | M | 131±4 | 73±11 | 26±6 | 49±13 | 66±7 |
| C57Bl/6 | 11 | F | 149±5 | 89±18 | 23±6 | 176±6 | 54±6 |
| BALB/c | 10 | F | 119±5 | 91±7 | 7±1 | 6±1 | 72±6 |
| DBA/2 | 13 | F | 137±6 | 97±10 | 8±1 | 7±2 | 101±8 |
| SJL/J | 14 | F | 101±5 | 55±13 | 6±4 | 5±2 | 136±12 |
| C3H/HeJ | 12 | F | 119±7 | 58±9 | 4±1 | 4±1 | 95±8 |

* Infected with 150 $L_3$ on day 0.

\+ Given 200 $L_3$ on days −22 and −10. Challenged with 150 $L_3$ on day 0. Mice killed and counts performed on day 5.

‡ Abbreviations: M = Male, F = Female.

** The intensity of the granulomatous reaction varied and was ranked as: +, CBA/H, A/J. ++ C57Bl/6, BALB/c, DBA/2, +++, SJL/J, C3H/HeJ.

\# Arithmetic mean ± SE. Each mean was determined from 4-5 naive mice or 8-10 preinfected mice.

significantly more resistant than C57Bl/6 mice ($P < 0.001$).

There were also marked differences in the numbers of adult worms present in immunised mice. For instance, male CBA/H mice accumulated adult worms arising from the immunising infections whereas male A/J and female C57Bl/6 mice had apparently lost some of the adult parasites. In contrast to these 3 groups, however, the number of adult worms found in females of the remaining strains was < 2.5% of the number present in the CBA/H males, and in each of these strains the residual worms were found in the distal portion of the small intestine rather than in their usual position in the duodenum.

The number of granulomata also varied between the groups of mice studied and was closely correlated with the number of cysts ($r = -0.797$; $0.02 < P < 0.05$) observed in immunised mice (Table 3.3). For example, those mice which had the greatest number of granulomata (e.g. female BALB/c, DBA/2, SJL/J and C3H/HeJ) had the least number of both encysted larvae as well as adult worms. Furthermore, the granulomata in these mice were noticeably more intense than those in the immunised CBA/H or A/J male mice (Footnote, Table 3.3). Because of this correlation between numbers of granulomata and resistance to challenge, the ratio of cyst frequency to granuloma frequency was analysed as a possible index of immunity. It can be seen in Table 3.4 that the cyst/granuloma (C:G) ratio was largest (0.43-1.5) in those mice which exhibited the least resistance. In contrast the C:G ratio for the mice which developed good

Table 3.4

Statistical analysis of the ratio of cysts to granulomata in the inbred strains of immunised mice

| Mice* Strain | Sex | No. | C:G+ ratio ± SE | Probability‡ VS.CBA/H | VS.C57Bl/6 |
|---|---|---|---|---|---|
| CBA/H | M | 8 | 1.51 ± 0.82 | - | 0.02<P<0.05 |
| A/J | M | 10 | 0.43 ± 0.11 | N.S.# | N.S. |
| C57Bl/6 | F | 10 | 0.58 ± 0.23 | N.S. | - |
| BALB/c | F | 9 | 0.096 ± 0.02 | P<0.002 | 0.002<P<0.02 |
| DBA/2 | F | 10 | 0.075 ± 0.01 | P<0.002 | P<0.002 |
| SJL/J | F | 10 | 0.032 ± 0.06 | P<0.002 | P<0.002 |
| C3H/HeJ | F | 10 | 0.036 ± 0.01 | P<0.002 | P<0.002 |

* Same mice as Table 3.3

\+ Abbreviations, C = Cysts, G = Granulomata, M = Male, F = Female.

‡ Determined by the 'U' test of Mann and Whitney.

\# Not significant, P>0.05

protective immunity was approximately 0.035. Additionally, the index was closely correlated with the ability of immunised mice to eliminate adult worms (r=0.972). Statistical analysis of the C:G ratios in Table 3.4 demonstrate that the C:G ratio could be used as an index of immune status. It is perhaps relevant that the mesenteric lymph nodes of the animals with low C:G ratios were greatly enlarged.

5. Development of resistance in inbred male and female mice.

The above study showed that immunised male and female outbred LACA mice did not differ in their ability to resist a challenge dose of $L_3$. However, in the above experiment the inbred mice which developed the best resistance were all females indicating that in the inbred strains of mice, females may develop better resistance than males.

To resolve this point, male and female mice of CBA/H and C3H/HeJ strains were immunised and challenged according to the schedule described in Fig. 3.1. As can be seen from Table 3.5 naive mice of either sex and of either strain were equally susceptible to a primary infection. The results using immunised mice (Table 3.5) show that in both strains, male mice exhibited poorer immunity than did females as judged by all the three parameters used to measure resistance. The C3H/HeJ male and CBA/H female mice exhibited a similar degree of resistance whilst C3H/HeJ females were the most resistant, particularly in their ability to eliminate adult worms. The values of the C:G ratios (Table 3.5)

Table 3.5

Susceptibility of naive and immunised male and female mice to infection by N. dubius

| Mice* | | Naive mice+ | Immunised (preinfected) mice‡ | | | |
|---|---|---|---|---|---|---|
| Strain | Sex | No. cysts (day 5) | No. cysts | No. adults | No. Granulomata | C:G** ratio |
| CBA/H | M | 255 ± 10# | 32 ± 5 | 268 ± 9 | 11 ± 2 | 3.919±0.798*** |
| CBA/H | F | 241 ± 7 | 8 ± 1 | 210 ± 9 | 41 ± 4 | 0.235±0.053 |
| C3H/HeJ | M | 237 ± 11 | 15 ± 2 | 217 ± 13 | 96 ± 7 | 0.160±0.022 |
| C3H/HeJ | F | 253 ± 8 | 7 ± 1 | 68 ± 10 | 108 ± 13 | 0.069±0.014 |

* Mice aged 11 weeks on day 0.

\+ Infected with 250 $L_3$ on day 0, cysts counted on day 5 (5 mice/group).

‡ Given 200 $L_3$ on days -22 and -10, challenged with 250 $L_3$ on day 0 (10 mice per group). Mice killed and cyst, adult worm and granulomata counts performed on day 5.

** Abbreviations; see Table 3.3

\# Arithmetic mean ± SE.

*** Statistical analysis, 'U' test of Mann and Whitney.

C3H/HeJ male vs C3H/HeJ female, $0.002<P<0.01$
CBA/H male vs CBA/H female, $P<0.002$

C3H/HeJ male vs CBA/H male $P<0.002$
CBA/H female vs C3H/HeJ female $P<0.002$

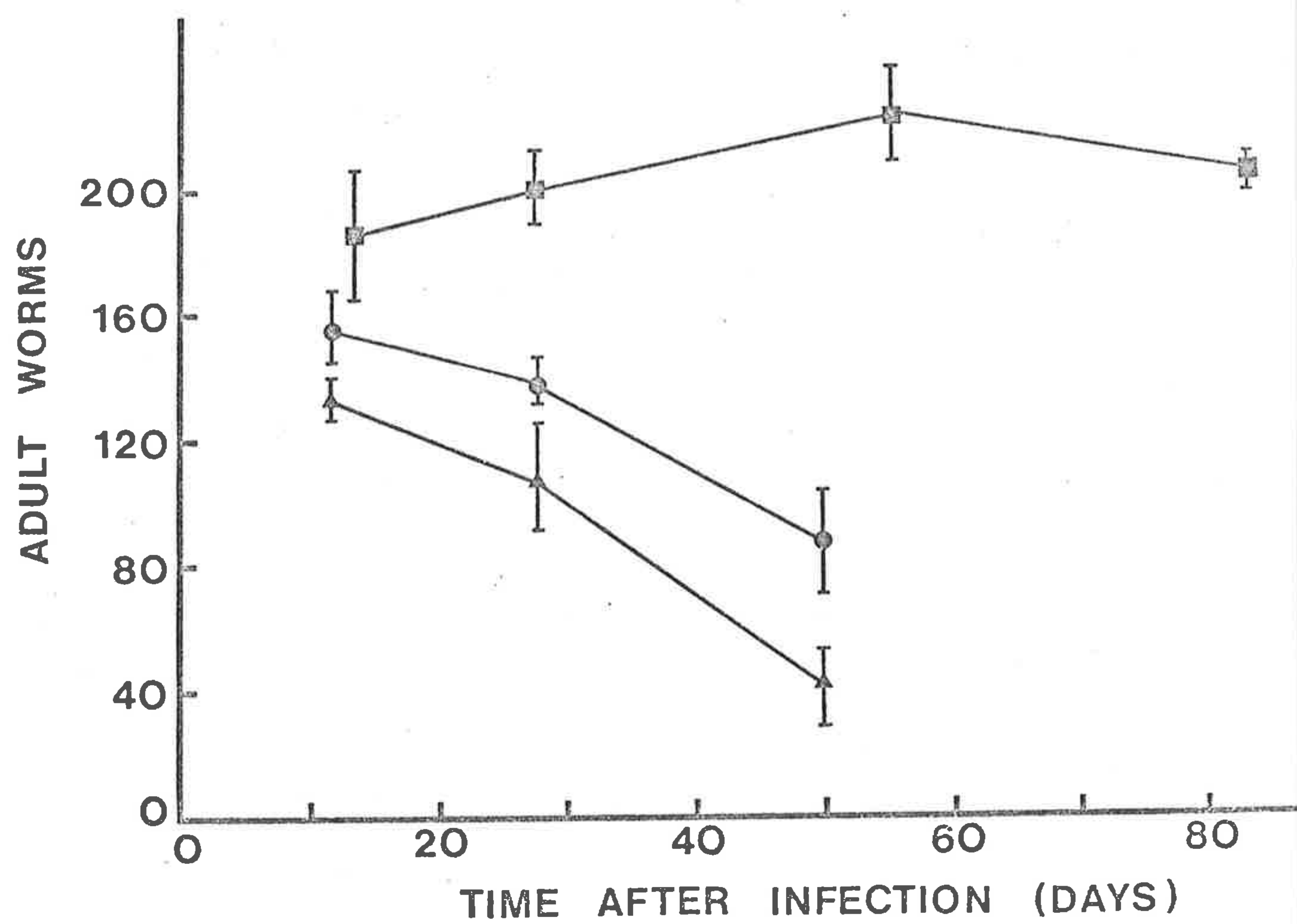
for the immunised mice of either strain show clearly that males were significantly less efficient than females in killing encysting larvae. The same conclusion can be made regarding elimination of adult worms. Comparing strains, CBA/H mice responded less well than C3H/HeJ mice of the same sex.

6. Survival of adult worms in mice following a primary infection

The differences in the number of adult worms observed in different strains of mice 19 days after a single infection (Table 3.3) suggested that the worms may be slowly eliminated. To examine this phenomenon in more detail, groups of female CBA/H, female C3H/HeJ and male and female LACA mice were infected with 200 $L_3$. On day 12 (CBA/H, C3H/HeJ), day 14 (LACA), day 28 (CBA/H, C3H/HeJ, LACA), day 50 (CBA/H, C3H/HeJ) and days 56 and 84 (LACA), 5 mice of each strain were killed and adult worms counted. The results in Figure 3.2 show that there was a steady loss of worms from the C3H/HeJ and CBA/H mice. In contrast, no loss was observed from LACA mice of either sex. The half-life of the adult worms in C3H/HeJ mice was approximately 40 days and approximately 55 days (by extrapolation) in CBA/H mice. Granulomata were still evident on day 28 in the C3H/HeJ mice but not in the CBA/H or LACA mice suggesting that C3H/HeJ mice mount an inflammatory response of greater intensity than the other two strains.

Figure 3.2

Persistance of adult N. dubius in CBA/H (●——●), C3H/HeJ (▲——▲) and LACA mice (■——■) following a single oral administration of third stage larvae. Vertical bars represent two standard errors.


ADULT WORMS
200
160
120
80
40
0
0
20
40
60
80
TIME AFTER INFECTION (DAYS)

7. Survival of adult worms in mice given repeated administrations of $L_3$

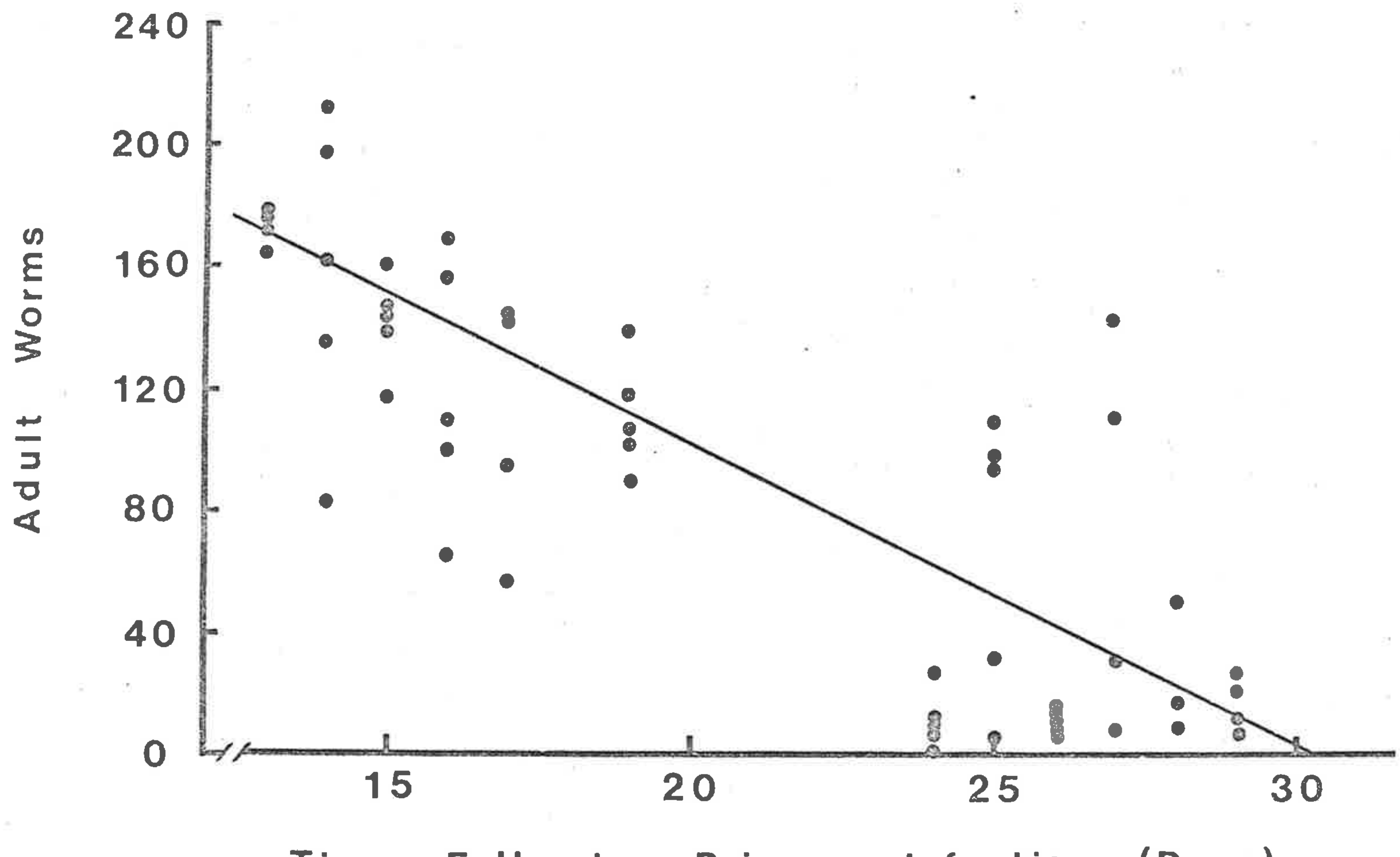
The loss of adult worms from some strains of immunised mice suggested that a type of self-cure phenomenon was involved, similar to that described by Cypess and Van Zandt, (1973) and for other host/parasite relationships (Stoll, 1929; Stewart, 1955). In order to investigate this possibility, a group of 54 female BALB/c mice received 200 $L_3$ on day 0. On day 13, 4 mice were killed and adult worms counted. Some of the remaining mice, all of which had received a further 200 $L_3$ on day 13, were killed in groups of 4-5 on days 14, 15, 16, 17, 19 and 24 after the primary infection. The remaining animals received one more administration of 200 $L_3$ (day 24) and, on days 25, 26, 27, 28 and 29, groups of 3-5 mice were killed and adult worms counted. The results are depicted in a scatter diagram (Figure 3.3). A regression analysis of the data indicated that adult worms were steadily lost from animals over the duration of multiple infections, 50% of the worms arising from the primary infection being lost by days 21-22. The steady loss of worms suggested that the mechanisms involved in worm expulsion in these mice differed from those involved in the explosive self cure phenomenon.

8. The loss of adult worms from mice which had either undergone a larval infection or which had received adult worms orally

Since it was apparent that adult worms were lost from mice following a primary larval infection it was of interest

Figure 3.3.

Persistance of adult N. dubius in BALB/c female mice dosed orally with third-stage larvae on days 0, 13 and 24. The regression line (correlation coefficient, -0.8145, n=54, $p < 0.001$) is shown.



240
200
160
120
80
40
0
Adult Worms
15
20
25
30
Time Following Primary Infection (Days)

to know whether adult worms transferred to mice which had not undergone a larval infection would be lost at a similar rate.

Twenty C3H/HeJ female mice received 150 $L_3$ orally on day 0. On each of days 7, 8 and 9 a further 20 age matched C3H/HeJ female mice received orally a dose of approximately 50 adult worms. Five mice from each group were killed on days 12, 23, 40 and 60 and the adult worms counted.

The results in Figure 3.4 show that there was some loss of worms from the mice which had undergone a larval infection (P=0.048, 'U' test, day 0 versus day 60) and a slightly lower loss from the mice which received only adult worms orally (P=0.063,'U'test, day 0 versus day 60). It is worth noting that the rate of loss in this experiment was less than that in other experiments. The reasons for this and the factors controlling the rate of loss of adult worms remain to be fully investigated.

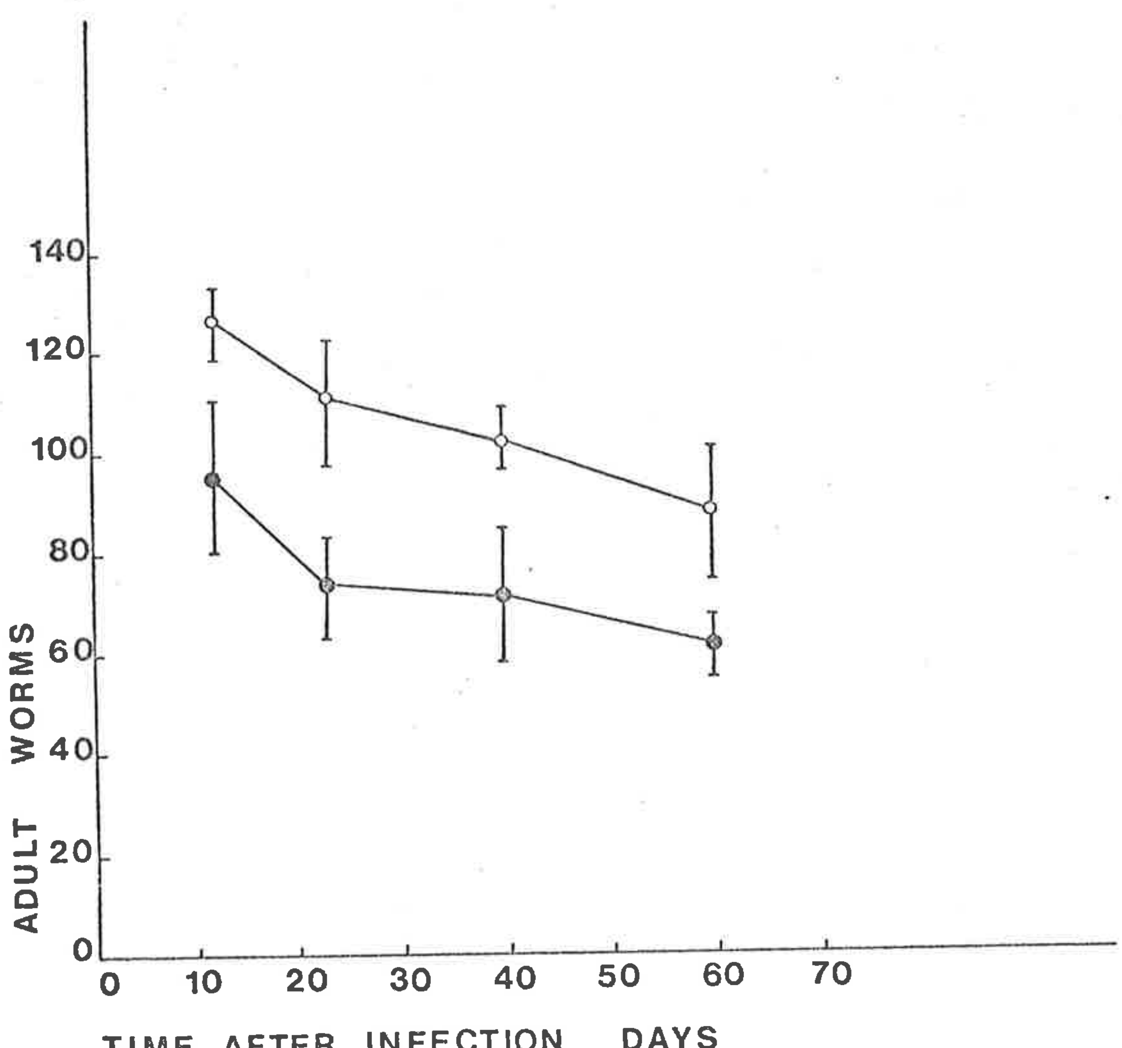
9. The development of resistance to N. dubius in (C3H/HeJ$_♀$ × CBA/H$_♂$) $F_1$ and (CBA/H$_♀$ × C3H/HeJ$_♂$) $F_1$ mice

Since such marked differences in the development of resistance between C3H/HeJ mice, which developed good resistance and CBA/H mice which developed poor resistance were apparent , further investigations were carried out to determine if the capacity to develop resistance to this infection was a dominant or recessive genetic trait.

In the initial experiment male mice of the CBA/H and C3H/HeJ strain and (C3H/HeJ$_♀$ × CBA/H$_♂$) $F_1$ hybrid male and

Figure 3.4

The loss of adult worms from mice which had either undergone a larval infection or which had received adult worms orally. Open circles, mice received one infection of 200 $L_3$ on day 0; closed circles, mice received 3×50 adult worms orally on days 7,8 and 9. Vertical bars represent 2 standard errors.



ADULT WORMS
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
TIME AFTER INFECTION DAYS

female mice were immunised and challenged according to the schedule shown in Figure 3.1.

The results are shown in Table 3.6. They indicate that the $F_1$ hybrid mice developed a degree of resistance similar to that of the CBA/H mice, that is the strain of mouse which developed the poorest resistance. This is indicated by the relatively high cyst numbers and the poor granuloma forming ability of the $F_1$ mice. The similarities between the CBA/H and $F_1$ mice are further reflected in the C:G ratios.

The second experiment was designed to study the development of resistance in ($C3H/HeJ_♀$ × $CBA/H_♂$) $F_1$ mice and ($CBA/H_♀$ × $C3H/HeJ_♂$) $F_1$ mice. Male and female mice of each parent were immunised and challenged according to the same schedule used previously.

Upon analysis the data in Table 3.7 showed that there was a highly significant difference ($p < 0.002$) between the C:G ratios of the 2 male $F_1$ hybrid groups but no significant difference between the female hybrid groups. The implications of this finding will be discussed later.

10. Conclusions

These studies have compared, for the first time the number of cysts, adult worms and granulomata in a wide range of commonly used inbred strains of mice and outbred LACA mice following 3 oral infections with third stage larvae. Upon analysis the ratio of cysts to granulomata proved to be a very sensitive index of resistance and

Table 3.6

The development of resistance to N. dubius in (C3H/HeJ × CBA/H) $F_1$ mice

| Mice[+] | | Naive Mice* | Pre-infected mice‡ | | | C:G[#] |
|---|---|---|---|---|---|---|
| Strain | Sex | Cysts | Cysts | Adults | Granulomata | |
| CBA/H | M | 169±4** | 60±6 | 309±36 | 12±2 | 5.4±0.9*** |
| C3H/HeJ | M | 172±7 | 17±4 | 223±28 | 89±9 | 0.2±0.05 |
| $F_1$ | M | 180±4 | 74±11 | 226±11 | 32±10 | 8.7±6.2 |
| $F_1$ | F | 177±3 | 42±10 | 291±16 | 62±9 | 0.8±0.2 |

\+ Aged 7 weeks on day -24

* Given 200 $L_3$ on day 0

‡ Given 200 $L_3$ on days -24, -12 and 0

\# Abbreviations, C ≡ cyst, G ≡ granulomata, M = male
F = female

** Arithmetic mean, ± SE. Parental strains, 5 mice per group; $F_1$, 6 mice per group

*** Analysis of C:G ratios ('U' test of Mann and Whitney)

| | | |
|---|---|---|
| CBA/H♂ vs $F_1$♂ | | P = 0.165 not significant |
| C3H/HeJ♂ vs $F_1$♂ | | P = 0.002 |
| CBA/H♂ vs C3H/HeJ♂ | | P = 0.002 |

Table 3.7

The development of resistance to *N. dubius* in

CBA/H, C3H/HeJ,

(C3H/HeJ♀×CBA/H♂)$F_1$ and (CBA/H♀×C3H/HeJ♂)$F_1$ mice

| Mice[+] | | Naive Mice[‡] | Pre-infected mice* | | | |
|---|---|---|---|---|---|---|
| Strain | Sex | Cysts±SE Day 5 | No. | Cysts±SE Day 5 | Granulomata ±SE Day 5 | C:G[#]±SE |
| CBA | M | 115±6 | 10 | 35±5 | 18±4 | 3.1±0.9 |
| C3H | M | 93±2 | 9 | 12±3 | 70±10 | 0.21±0.06 |
| C3H×CBA | M | 108±9 | 10 | 38±4 | 27±7 | 2.5±1** |
| CBA×C3H | M | 116±8 | 9 | 8±2 | 61±7 | 0.15±0.04 |
| CBA | F | 121±6 | 10 | 23±4 | 46±10 | 0.84±0.29 |
| C3H | F | 108±11 | 7 | 8±2 | 80±17 | 0.14±0.05 |
| C3H×CBA | F | 121±6 | 8 | 12±2 | 55±8 | 0.27±0.07 |
| CBA×C3H | F | 114±6 | 5 | 25±5 | 44±15 | 1.16±0.1 |

\+ Aged 9 weeks on day -20

‡ Groups of 4-5 mice, 150 $L_3$ on day 0

* Mice given 200 $L_3$ days -20, -10; 150 $L_3$ on day 0

\# Abbreviations M = male, F = female, C = cyst, G = granulomata, SE = standard error.

** Statistical analysis C3H×CBA♂ vs CBA×C3H♂ $P<0.002$
C3H×CBA♀ vs CBA×C3H♀ $P=0.217$

'U' test of Mann and Whitney.

clearly showed the differences between strains.

It was also evident that in two of the inbred strains, female mice could develop better resistance than male mice. This was in contrast to the results found in outbred mice. Several of the inbred strains studied had the ability to rid themselves of adult worms following a primary infection whereas the LACA mice did not. This loss of adult worms also occurred in inbred mice which had received several administrations of third stage larvae.

Studies of the development of resistance of hybrids between mice which develop good resistance and mice which develop poor resistance have indicated that there were differences in the ability of the various hybrids to develop resistance to N. dubius. The implications of this will be considered later.

# CHAPTER 4

## *N. dubius* infection in LACA mice

1. Introduction
2. The fate of infective third stage larvae following challenge of immune mice
3. The role of the cysts and the adult worms in the induction of immunity and $IgG_1$ production
4. Granulomata formation around larvae entrapped in intestinal tissue
5. Conclusions

1. Introduction

Data presented in the previous chapter showed that LACA mice developed good resistance to reinfection following two previous exposures to living third stage larvae. This immunity, as assessed by cyst counts suggested that the mechanisms involved in the increased resistance, operated against the third stage larvae used in the challenge and not against the adult worms since resistant mice still carried worm burdens resulting from the immunising infections. The persistence of the adult worms in the LACA mice resembled, in some ways, the persistence of adult worms in some nematode infestations in man where, inspite of continuous exposure to infection, the adult worms remain in the intestine of the host.

However what is not clear is whether the immune mechanisms operate on the larvae prior to penetrating the mucosa or afterwards. Recently Jones and Rubin, (1974) have produced evidence indicating that the mechanisms of resistance to infection displayed by immunised mice depended upon the route used for immunisation. They state that orally-immunised mice expel the challenge larvae by a mechanism similar to that proposed by Panter, (1969b) whereas in mice immunised subcutaneously the immune mechanisms operate on the larvae during penetration of the small intestine.

2. The fate of third stage larvae following challenge of resistant mice

In order to obtain a better understanding of the

mechanisms of resistance in immunised mice there was a need to determine the fate of the challenge dose of $L_3$ which did not form cysts in resistant animals. To study this problem in detail, use was made of the formation of granulomata around a foreign body in the intestinal mucosa.

It was argued that if the larvae were expelled before penetration, then the number of granulomata seen in challenged immune mice should be no different from that observed in unchallenged immune mice. On the other hand, if the larvae penetrated the wall of the intestine, then the number of granulomata should be increased in the challenged immune mice by an amount bearing some relationship to the challenge dose. The following experiments were designed to differentiate between these two possibilities.

In the first experiment mice were immunised either orally or intraveneously by the administration of 3 doses of 200 $L_3$ given 14 days apart. Ten days after the final dose the mice received pyrantel pamoate orally, to clear adult worms arising from the immunising infections. Immunised mice were divided into 4 groups of 10 (A, B; immunised orally; C, D; immunised intravenously). Groups A and C were left unchallenged as granulomata controls. Groups B and D and a group of non-immunised control mice were given an oral challenge of 250 $L_3$.

Table 4.1 shows that mice immunised orally or intravenously and challenged orally had significantly more granulomata ($P<0.001$) than the respective control groups. This

Table 4.1

The incidence of granulomata in mice immunised either orally or intravenously following an oral challenge with $L_3$

| Group | Treatment | Challenge dose | Granulomata ±S.E. Day 21 | Adult worms ±S.E. Day 21 |
|---|---|---|---|---|
| Control | None | 250 $L_3$ | N.D.* | 222 ± 15 |
| A | 3×200 $L_3$ Orally | None | 152 ± 17 | 24 ± 6 |
| B | 3×200 $L_3$ Orally | 250 $L_3$ | 341 ± 27$^+$ | 26 ± 8 |
| C | 3×200 $L_3$ Intravenously | None | 77 ± 14 | 3 ± 1 |
| D | 3×200 $L_3$ Intravenously | 250 $L_3$ | 281 ± 9$^+$ | 27 ± 6 |

* N.D. = not done

\+ 't' test, A vs B $p < 0.001$, C vs D $p < 0.001$

increased incidence of granulomata corresponded closely with the number of larvae given in the challenge dose.

In a second experiment mice, immunised by the same schedule as above with the exception that the larvae were administered intraperitonealy, were divided into two groups (A, B) of 8 animals. Group A was left unchallenged as a granulomata control. Group B and a group of unimmunised control mice received an oral challenge of 200 $L_3$. It can be seen from the data in Table 4.2 that the immune mice had significantly more granulomata ($P<0.001$) following oral challenge than did the animals in the control group. The increased incidence of granulomata again corresponded closely with the number of larvae given in the challenge dose.

Finally mice were immunised subcutaneously by 2 injections of 2,300 $L_3$ with an interval of 21 days between injections. These mice were divided into 2 groups (A, B) of 10 animals. Group A was left unchallenged as a granulomata control. Group B and a group of unimmunised control mice received an oral challenge of 200 $L_3$. The results in Table 4.3 show that the challenged immunised mice had significantly more granulomata than did the unchallenged immune group. Once more, this increased incidence of granulomata corresponded closely with the number of larvae administered in the challenge dose.

3. The role of the cyst and adult worm stage of infection in the induction of immunity

In order to determine which stage of the cycle of infection stimulated the protective immune response, 2 groups

Table 4.2

The incidence of granulomata in mice immunised intraperitoneally following an oral challenge with $L_3$

| Group | Treatment | Challenge Dose | Granulomata ±S.E. Day 21 | Cysts ± S.E. Day 5 |
|---|---|---|---|---|
| Control | None | 200 $L_3$ | N.D.* | 173 ± 4 |
| A | 3×200 $L_3$ | None | 3 ± 1 | N.D. |
| B | 3×200 $L_3$ | 200 $L_3$ | 186 ± 21+ | 14 ± 1 |

* N.D. = not done

\+ 't' test, A vs B $p < 0.001$

Table 4.3

The incidence of granulomata in mice immunised subcutaneously following an oral challenge with $L_3$

| Group | Treatment | Challenge Dose | Granulomata ±S.E. Day 21 | Cysts±S.E. Day 5 | Adult worms ±S.E. Day 21 |
|---|---|---|---|---|---|
| Control | None | 200 $L_3$ | N.D.* | 173 ± 16 | N.D. |
| A | 2×2,300 $L_3$ | None | 31 ± 7 | N.D. | 0 |
| B | 2×2,300 $L_3$ | 200 $L_3$ | 179 ± 20$^{+}$ | N.D. | 13 ± 9 |

* N.D. = not done

\+ 't' test A vs B $p < 0.001$

of 10 mice were given 200 $L_3$ orally on day 0, and again on day 26. One group, (A) was given pyrantel orally on days 6, 7 and 8, and 31, 32 and 33 at a dose of 12 mg/kg body weight. The second group, (B) was not treated. On day 40 both groups together with an age and sex matched control group were challenged with 200 $L_3$ orally. Cysts, adult worms and granulomata were counted on day 45.

From the number of parasites shown in Table 4.4 it was clear that both groups, A and B developed the same high degree of resistance. It appeared possible that the group which had no adult worms (group A) was able to mount a marginally better inflammatory response as evidenced by the significantly higher number of granulomata. This experiment shows that good protective immunity may be stimulated by the larval stage of infection and the presence of adult worms was not necessary for the induction of resistance.

5. Granulomata formation around larvae entrapped in the intestinal tissue

In order to obtain further evidence that larvae entrapped in the intestinal mucosa resulted in a cellular infiltration and subsequent granulomata formation photographs were taken of the various stages of granulomata development in the mucosa of resistant and normal mice on day 5 following oral challenge with $L_3$. The results are shown in Figure 4.1. The first photograph shows a normal cyst as it appears in an animal following a primary infection. The second photograph shows a cyst from a challenged resistant animal. Although there is a large infiltration of cells into the

Table 4.4

The stimulation of resistance by the cyst stage of the infectious cycle

| Naive Mice[+] | Preinfected mice | | | | |
|---|---|---|---|---|---|
| Cysts | Group | Treatment | Cysts | Adult worms | Granulomata |
| 107 ± 6* | A | Pyrantel | 9 ± 3 | 0 | 156 ± 9 |
| | B | None | 10 ± 4 | 128 ± 16 | 92 ± 14‡ |

\+ Aged 7 weeks at day 0

* Arithmetic mean ±S.E.. All groups contained 10 mice.

‡ A vs B (granulomata) 't' test $0.002 < p < 0.001$

tissue surrounding the worm, the cyst appears intact. The third photograph shows a smaller worm in which development appears to have been retarded. The worm also appears to be broken up. The fourth photograph shows an area of marked cellular infiltration, only small sections of the parasite are visible. The final stage of the destruction of the worm is shown in the 5th photograph where the residual pieces of worm are almost invisible under the huge cellular infiltration. The result of this massive cellular infiltration is shown in the 6th photograph where the lesion resulting from earlier immunising infections has become a calcified, hard yellowish nodule in the intestinal wall. In immune animals these take up to 100 days to regress. Since photographs 2 - 6 were taken in the same group of animals on the same day the fate of the worm probably depends on:-

(a) the time and site of penetration of the mucosa after challenge.

(b) the rate of mobilization of cells by the host.

(c) the intensity of the hosts immune response against the invading $L_3$.

Because of these variations some larvae might be expected to develop to maturity in immune animals.

5. Conclusions

Data presented here clearly showed that the immune mechanisms operated on the larvae after it penetrated the wall of the intestine. The larvae always penetrated the

Figure 4.1

Granulomata formation around larvae trapped in the intestinal tissue.

1. Cyst in intestinal wall of a normal mouse resulting from a primary infection.

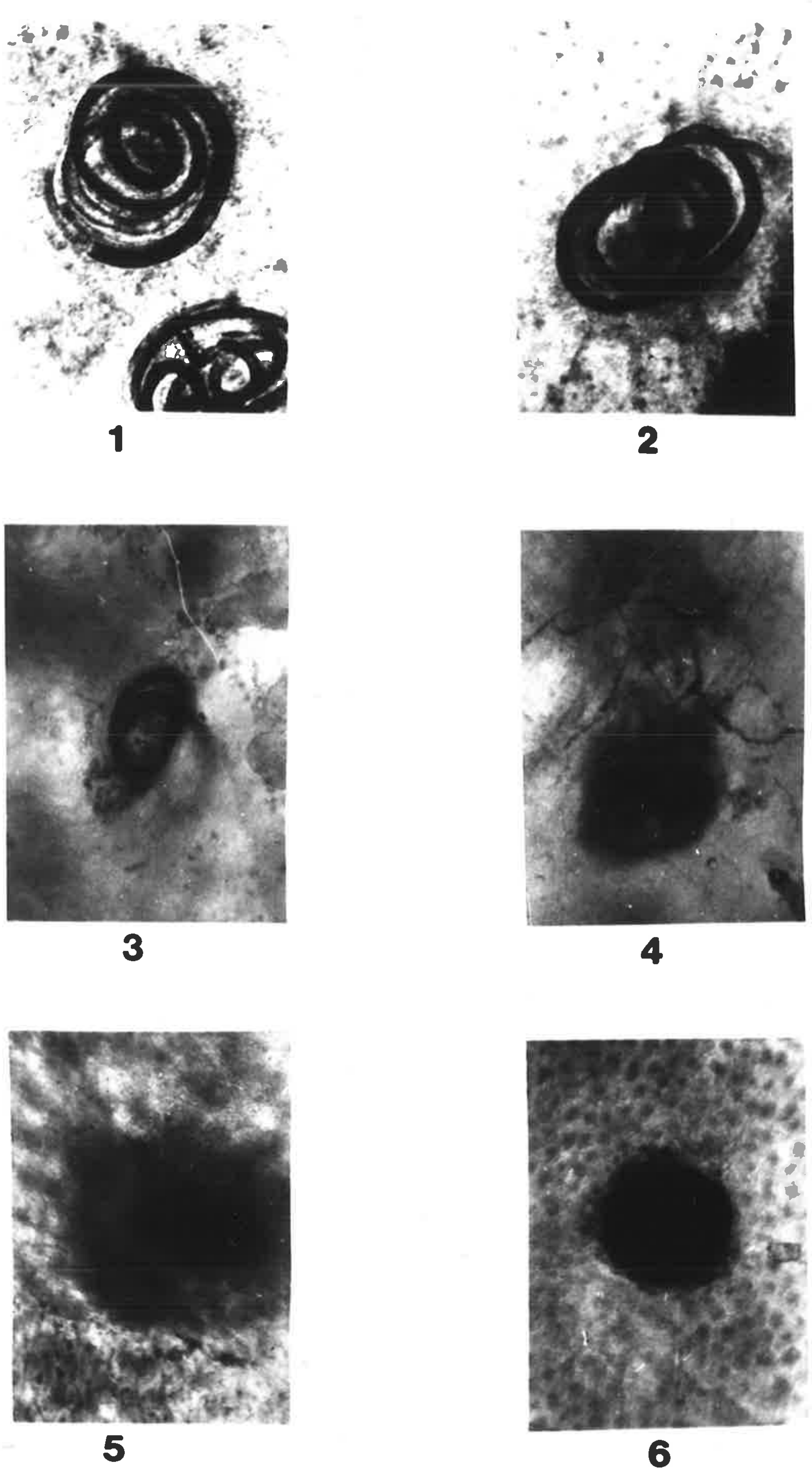
2. Cellular infiltration around a cyst in an resistant mouse.

3. Intense cell infiltration around a fragmented, incomplete worm.

4. Intense cell infiltration with only small pieces of worm visible.

5. Early stage of granuloma development with more intense cell infiltration, no visible worm fragments.

6. Solid, white granuloma with calcification commenced.


1
2
3
4
5
6

wall of the intestine in mice which were highly resistant regardless of the route used for immunisation. No evidence was obtained that mice immunised orally expelled the larvae before penetration. Furthermore, the presence of adult worms was not necessary to stimulate immunity, however they may have had some effect on the ability of the mice to mount an inflammatory response.

The series of photographs, expecially nos. 2 - 5 suggest strongly that in immune mice, the cellular infiltration around the larvae leads to their destruction as is evident by the fragments of worms seen inside the granulomata.

# CHAPTER 5

## Cell and Immunoglobulin changes and the onset of immunity in mice to *N. dubius*

1. Introduction
2. Changes in cell levels following multiple oral administrations of $L_3$
3. Changes in cell levels following multiple intravenous administrations of $L_3$
4. Changes in total cell numbers following the intraperitoneal injection of $L_3$
5. Occurrence of eosinophils in the bone marrow following a single infection by *N. dubius* $L_3$
6. Changes in serum immunoglobulin isotype levels following either multiple oral or intravenous administration of $L_3$
7. Changes in serum $IgG_1$ and $IgG_{2a}$ concentration following a single infection of $L_3$
8. The role of adult worms in the stimulation of $IgG_1$ production
9. Time course for the development of immunity following intravenous and oral immunisation with third stage larvae.
10. Enhanced protection of infected mice following the administration of serum from immune mice
11. Serum $IgG_1$ and $IgG_{2a}$ concentrations in infected mice of inbred strains
12. Conclusions

1. Introduction

It has been shown that LACA mice infected twice with 200 $L_3$ develop a high degree of protective immunity to subsequent infections (Chapter 3). The macrophage is believed to be an important component of this immunity as it has been shown to bind both in vivo and in vitro to $L_3$ impairing their infectivity. Binding in vitro has been shown to be dependent upon the presence of serum from immune mice, (Chaicumpa, Jenkin and Fischer, 1977; Chaicimpa and Jenkin, 1977). Eosinophils may also be involved in immunity since Jones and Rubin (1974), have reported that eosinophils come into intimate contact with the encysting larvae, forming a major part of the granulomatous lesions which develop around damaged worms. However, the eosinophil was not considered to be one of the cells involved in killing the larvae by Chaicumpa and Jenkin, (1977).

In view of this it seemed important to study in more detail, the changes in cell type and number and in immunoglobulin levels which occurred as a result of multiple infections in mice and to relate, if possible these changes to the development of resistance to this infection. It was also important to determine whether different routes of immunisation induced different changes in the cell and immunoglobulin levels since Jones, (1974) has claimed that the different routes of immunisation resulted in different mechanisms of resistance.

2. Changes in cell levels following multiple oral administrations of $L_3$

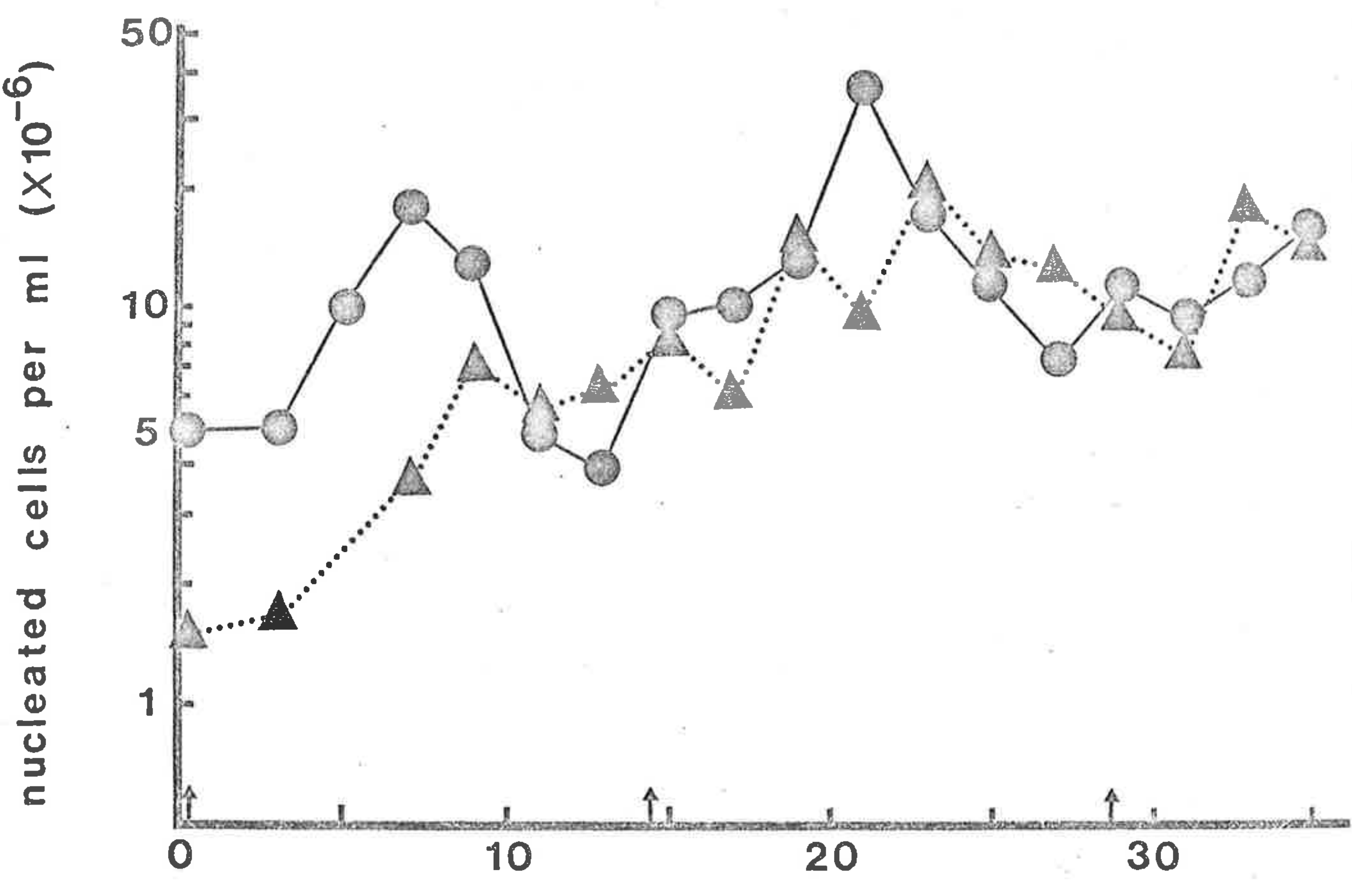
A group of mice received 3 doses of 200 $L_3$ orally 14 days apart. At intervals of 2-3 days total and differential leukocyte counts were carried out on the blood and peritoneal washouts of 5 mice selected at random.

The changes in the total number of leukocytes in the blood and peritoneal cavity are shown in Figure 5.1. The standard deviations are shown in Table 5.1. It can be seen that the leukocyte levels, particularly in the blood, peaked 6-8 days after each dose, at the time when the emerging juvenile worms were migrating back into the intestinal lumen. A marked increase in the number of peritoneal exudate cells was apparent during the initial infection. In contrast to the number of leukocytes in the blood which peaked at days 6-8 and returned to normal levels by days 11-12, peritoneal exudate cell numbers were still elevated at the time of the second immunising infection (day 14). Cell numbers in both the blood and the peritoneal cavity then increased to approximately $1\text{-}2 \times 10^7$ cells/ml (days 18-26) and declined slowly until the third dose of larvae was given on day 28.

The initial change in the number of blood leukocytes is due mainly to a rise in the number of lymphocytes and neutrophils (Fig. 5.2), whereas in the peritoneal cavity, macrophages as well as lymphocytes and neutrophils increased in number (Fig. 5.3). Although the relative increase of lymphocytes and neutrophils in the peritoneal cavity exceeded that of macrophages ($\geq$ 20 fold compared to 2-4 fold), the

Figure 5.1

Mean total (5 mice) leukocyte count in the blood and peritoneal washouts of mice following multiple oral infections with N. dubius $L_3$. Mice were infected (↑) with 200 $L_3$ on day 0 and again on days 14 and 28. Blood count, ●———● ; peritoneal washout, ▲·········▲. Standard deviations shown in Table 5.1.



nucleated cells per ml (X10⁻⁶)
50
10
5
1
0
10
20
30
time following initial infection (days)

Table 5.1

The means and standard deviations of the total number of leukocytes in the peritoneal cacity and blood following oral infections with N. dubius $L_3$.

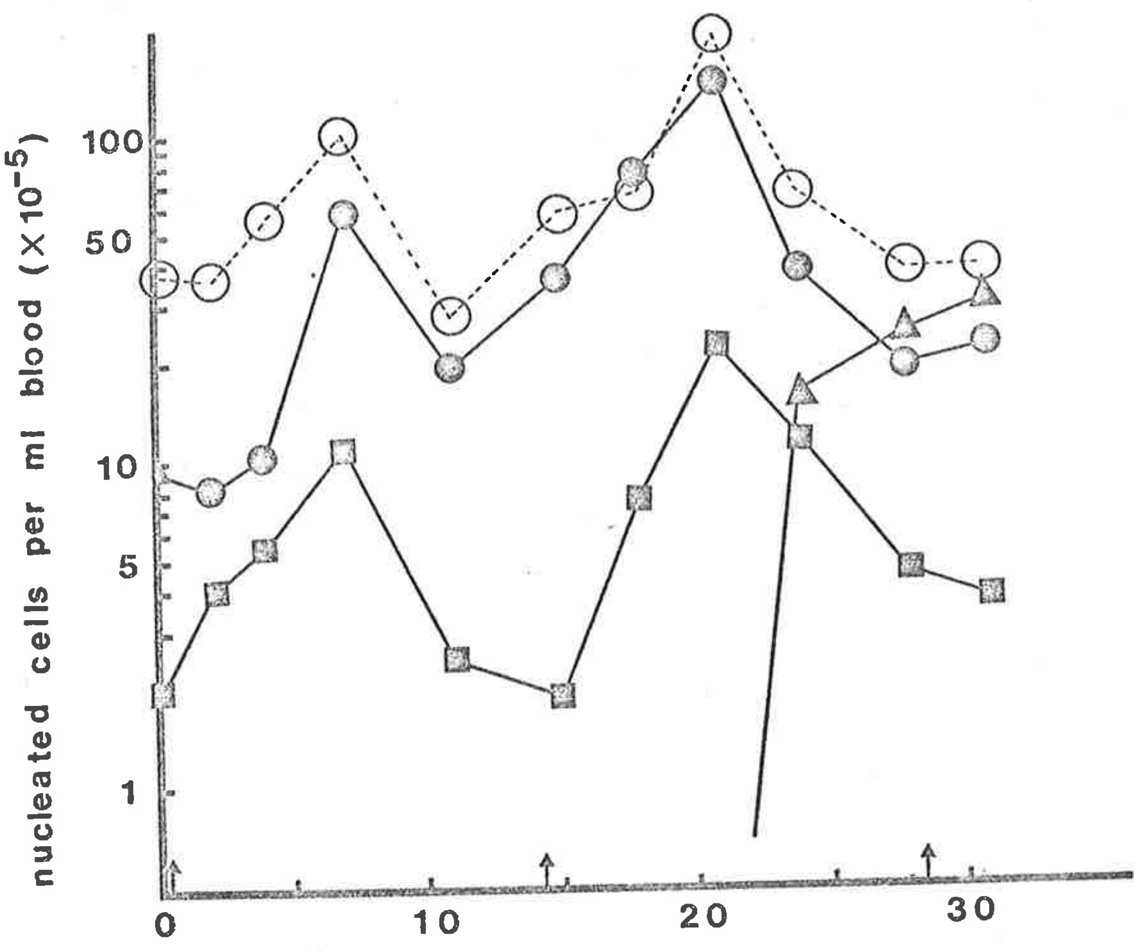
| Day following initial infection | Mean+ no. cells/ml ± S.D. (×10$^{-6}$) | |
|---|---|---|
| | Peritoneal exudate cells | Blood leukocytes |
| 0* | 1.5 ± 0.4 | 4.9 ± 0.5 |
| 3 | 1.6 ± 0.3 | 5.2 ± 1.7 |
| 5 | 2.6 ± 0.3 | 10.3 ± 4.8 |
| 7 | 3.7 ± 0.6 | 18.2 ± 7.7 |
| 9 | 7.1 ± 1.8 | 13.8 ± 5.2 |
| 11 | 5.5 ± 0.8 | 4.9 ± 1.8 |
| 13 | 6.1 ± 2.7 | 3.9 ± 0.7 |
| 15* | 8.5 ± 2.1 | 9.5 ± 1.1 |
| 17 | 5.8 ± 1.1 | 10.0 ± 2.6 |
| 19 | 14.8 ± 5.3 | 13.3 ± 3.9 |
| 21 | 9.2 ± 4.7 | 3.8 ± 1.6 |
| 23 | 20.6 ± 5.2 | 10.6 ± 1.1 |
| 25 | 13.9 ± 6.8 | 11.7 ± 5.0 |
| 27 | 12.3 ± 1.3 | 7.2 ± 2.6 |
| 29* | 9.1 ± 3.1 | 11.4 ± 5.3 |
| 31 | 7.3 ± 2.6 | 9.3 ± 2.1 |
| 33 | 17.0 ± 1.3 | 12.0 ± 4.2 |

\+ Arithmetic mean of 5 mice, same mice as in Fig. 5.1

* Administration of larvae.

Figure 5.2

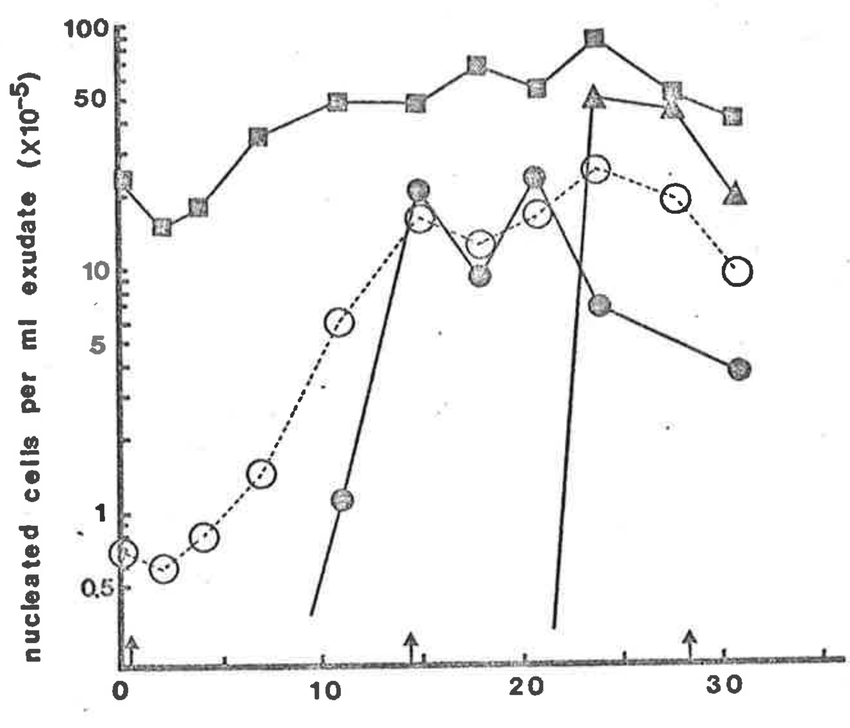
Differential analysis of leukocytes in the blood of mice following oral infections with *N. dubius*. Animals were given 200 $L_3$ (↑) on days 0, 14 and 28. Lymphocytes, o·········o ; Neutrophyls, ●——● , Monocytes, ■——■ ; Eosinophils, ▲——▲. These values are the mean of counts of smears from 5 mice determined initially as a percentage of the cells counted and related to the total leukocyte counts.



nucleated cells per ml blood (×10$^{-5}$)
100
50
10
5
1
0
10
20
30
time following initial infection (days)

Figure 5.3

Differential analysis of peritoneal exudate cells following oral infections with _N. dubius_. Mice were given 200 $L_3$ (↑) on days 0, 14 and 28. Lymphocytes, o---------o ; Neutrophils, ●———● ; Macrophages, ■———■ ; Eosinophils, ▲———▲ . See Figure 5.2 for procedure.



nucleated cells per ml exudate (X10$^{-5}$)
100
50
10
5
1
0.5
0
10
20
30
time following initial infection (days)

absolute number of macrophages was always 3 to 4 times that of the other cell types. However the most dramatic increase was that of the eosinophils. These cells were virtually absent from the peritoneal cavity and the blood until day 21 when they appeared suddenly, increasing within 1-2 days to a relatively constant level of $2\text{-}5\times10^6$ cells/ml which was maintained for a number of weeks following the third immunisation. Eosinophilia was absolutely dependent upon a second immunising infection and did not appear in mice carrying long term primary infections despite the presence of many adult worms in the small intestine.

3. Changes in cell levels following multiple intravenous administrations of $L_3$

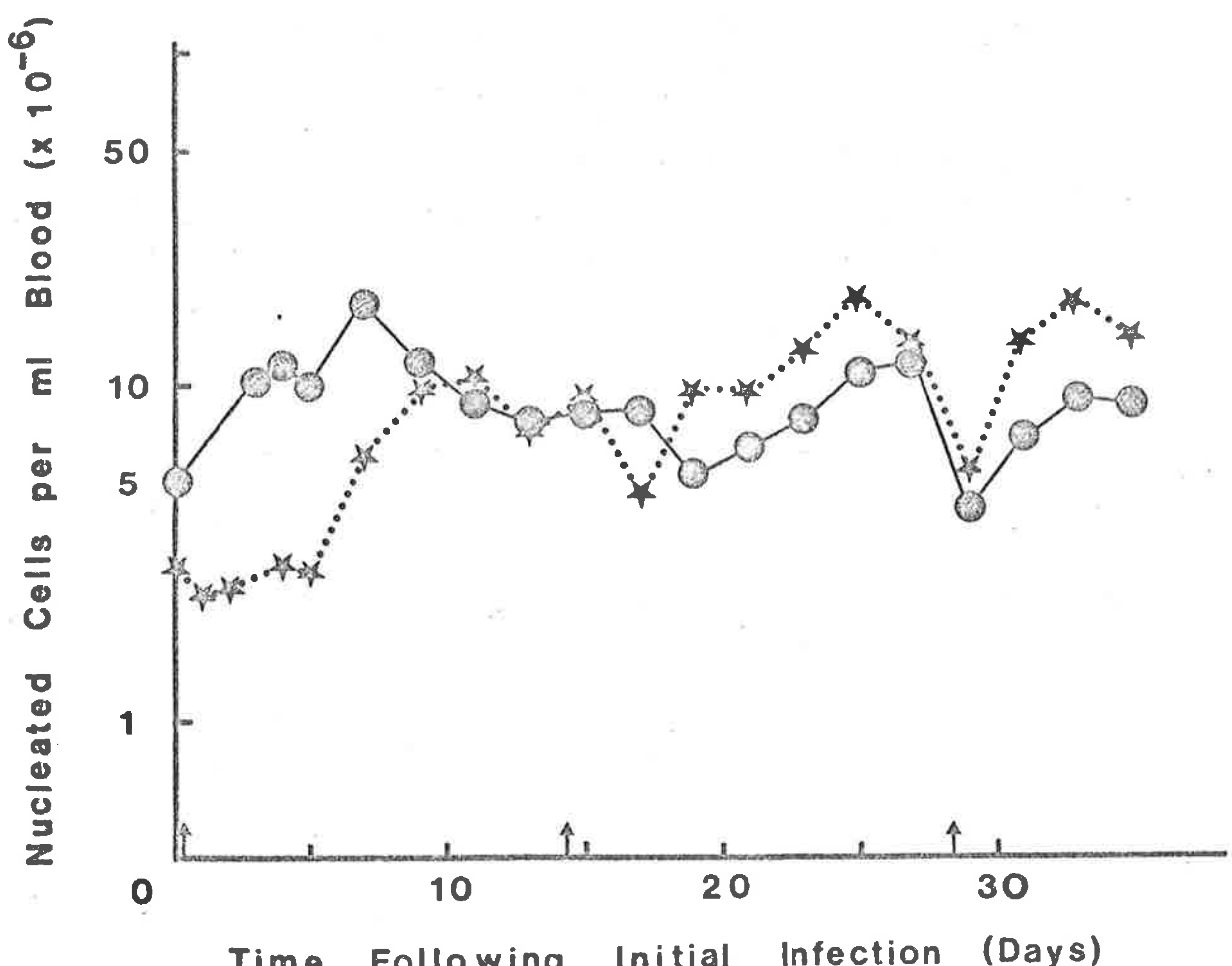
Larvae were administered to mice using the same schedule as described for mice infected orally. The changes in the number and type of blood leukocytes and peritoneal exudate cells were essentially the same as in mice immunised orally and are seen in Figures 5.4, 5.5 and 5.6 and Table 5.2. In view of using killed $L_3$ as a vaccine, mice were also immunised with 200 killed (freeze dried) $L_3$. No changes in cell levels could be detected, even following multiple injections of killed $L_3$.

4. Changes in total cell numbers following the intraperitoneal injection of $L_3$

Since mice which received 3 doses of $L_3$ intraperitoneally developed good immunity it was of interest to know whether they underwent similar changes in leukocyte number as did mice

Figure 5.4

Mean total (5 mice) leukocyte count in the blood and peritoneal washouts of mice following multiple intravenous infections with N. dubius $L_3$. Mice were infected (↑) with 200 $L_3$ on day 0 and again on days 14 and 28. Blood count, (●——●); peritoneal washout, (★·······★). Standard deviations shown in Table 5.2.



Nucleated Cells per ml Blood (x 10$^{-6}$)
50
10
5
1
0
10
20
30
Time Following Initial Infection (Days)

Table 5.2

The mean and standard deviation of total number of leukocytes in the peritoneal cavity and blood following intravenous injections of *N. dubius* $L_3$

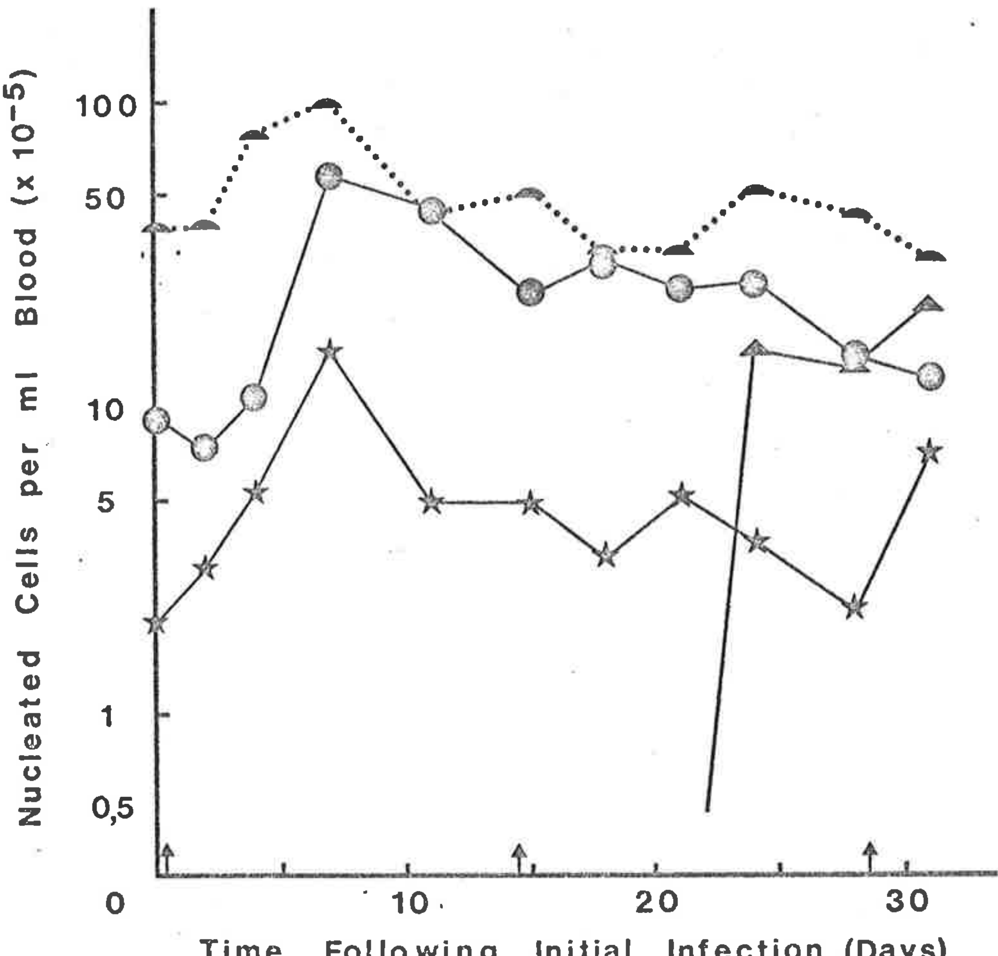
| Day following initial infection | Mean[+] no. cells/ml ± S.D. ($\times 10^{-6}$) | |
|---|---|---|
| | Peritoneal exudate cells | Blood leukocytes |
| 0* | 2.9 ± 1.1 | 5.1 ± 0.6 |
| 1 | 2.5 ± 0.5 | Not done |
| 2 | 2.5 ± 0.3 | Not done |
| 3 | Not done | 10.4 ± 2.5 |
| 4 | 2.9 ± 0.4 | 11.6 ± 3.5 |
| 5 | 2.8 ± 0.4 | 9.2 ± 7.4 |
| 7 | 6.0 ± 1.2 | 17.9 ± 11.1 |
| 9 | 9.6 ± 2.5 | 11.6 ± 4.8 |
| 11 | 10.3 ± 2.4 | 8.1 ± 1.6 |
| 13 | 7.1 ± 1.0 | 7.6 ± 2.0 |
| 15* | 8.9 ± 3.4 | 8.1 ± 3.0 |
| 17 | 4.6 ± 0.5 | 8.2 ± 3.8 |
| 19 | 9.8 ± 2.8 | 5.1 ± 1.6 |
| 21 | 9.1 ± 3.3 | 6.3 ± 2.3 |
| 23 | 12.3 ± 4.9 | 7.6 ± 2.5 |
| 25 | 17.6 ± 2.8 | 10.6 ± 3.5 |
| 27 | 13.1 ± 2.7 | 11.6 ± 2.3 |
| 31* | 13.0 ± 3.1 | 6.8 ± 1.6 |
| 33 | 17.1 ± 2.3 | 8.9 ± 1.9 |
| 35 | 13.7 ± 2.9 | 8.3 ± 2.5 |

\+ Arithmetic mean of 5 mice, same mice as Fig. 5.4

* Administration of larvae.

Figure 5.5

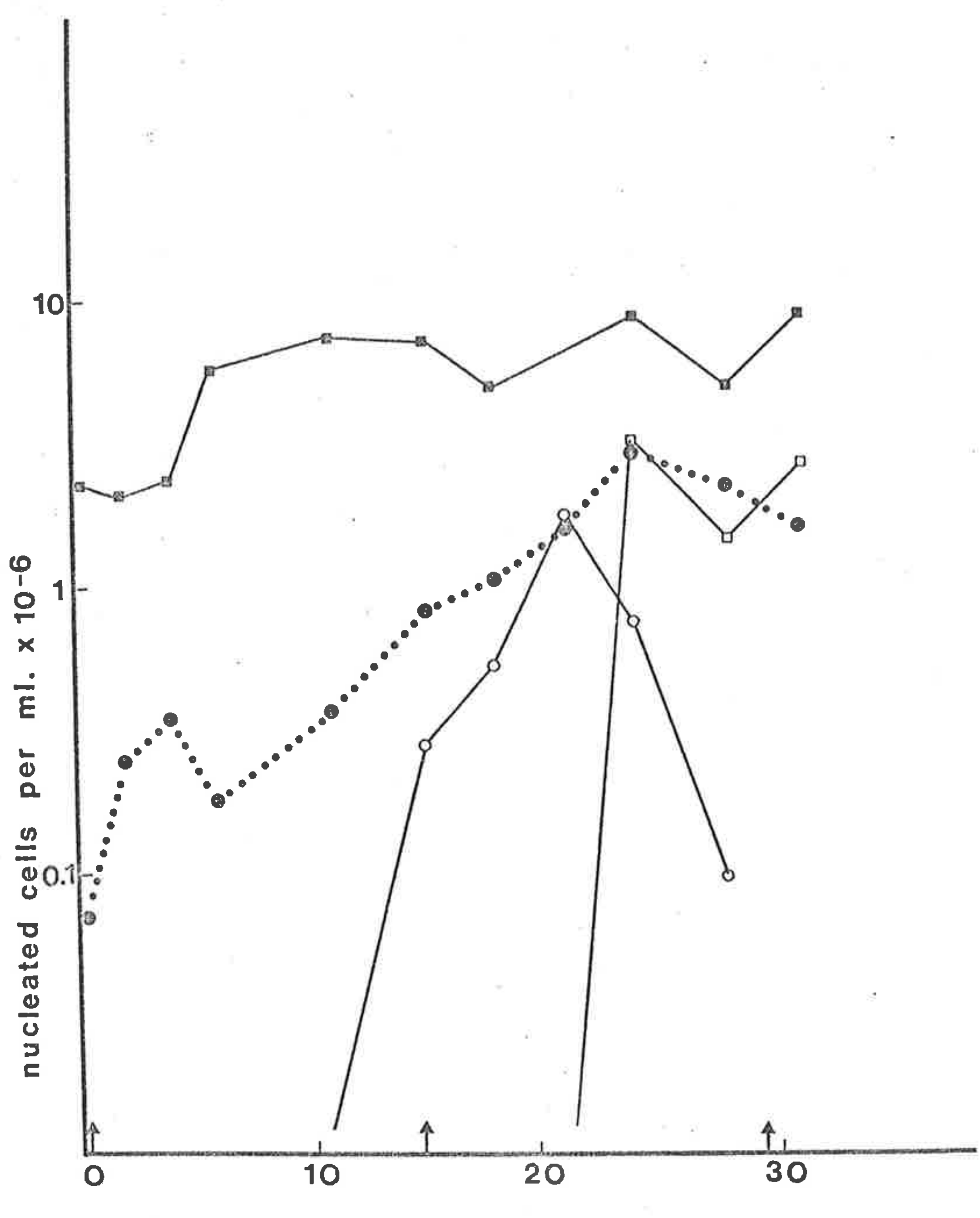
Differential analysis of leukocytes in the blood of mice following intravenous infection with N. dubius. Animals were given 200 $L_3$ on days 0, 14 and 28 (↑) Lymphocytes, ▲.............▲; Neutrophils, ★———★; Monocytes, ●———●; Eosinophils, ▲———▲. For procedure, see Figure 5.2.



Nucleated Cells per ml Blood (x 10$^{-5}$)
100
50
10
5
1
0,5
0
10
20
30
Time Following Initial Infection (Days)

Figure 5.6

Differential analysis of peritoneal exudate cells following intravenous administrations of N. dubius. Mice received 200 $L_3$ (↑) on days 0, 14 and 28. Macrophages (■——■), Lymphocytes (●·········●), Neutrophils (○——○), Eosinophils (□——□). See Figure 5.2 for procedure.



10
1
0.1
nucleated cells per ml. x 10-6
0
10
20
30
time following initial infection days

infected orally and intravenously. A group of mice received $L_3$ intraperitoneally using the same schedule as described for oral administration. At intervals of 2-3 days total leukocyte counts were carried out on the blood and peritoneal washouts of 5 mice selected at random. The results are shown in Figure 5.7 and the standard deviations in Table 5.3.

The peritoneal exudate cells increased in number immediately following immunisation and plateaued on day 17 at $1\text{-}2 \times 10^7$/ml There were only minor fluctuations in the cell levels for the remainder of the experimental period. In contrast, the leukocytes in the blood peaked consistently at 10-14 days following each dose of larvae, slowly declining thereafter to near normal levels. It is known that some of the larvae injected intraperitoneally do encyst in the parenchymal tissue, (Baker, 1962) and the peaks of blood leukocytes may be related to the release of products of parasite origin from these trapped, encysted larvae which are not able to develop into adult worms.

5. Occurrence of eosinophils in bone marrow following a single infection of N. dubius $L_3$

Since only a second infection by $L_3$ resulted in the appearance of eosinophils in the blood and peritoneal cavity it was important to determine the effect of a single infection on the numbers of eosinophils in the bone marrow.

A group of mice was infected orally with 200 $L_3$ and 13 days later the bone marrow was collected. Total leukocyte and eosinophil counts were carried out on cells from these and uninfected control mice. The results, (Table 5.4), show

Figure 5.7

Mean total (5 mice) leukocyte count in the blood and peritoneal washout of mice following intraperitoneal injections of *N. dubius* $L_3$. Mice received 200 $L_3$ (↑) on days 0, 14 and 28.

White blood cells ●——●

Peritoneal cells ■——■

Standard deviations shown in Table 5.3.

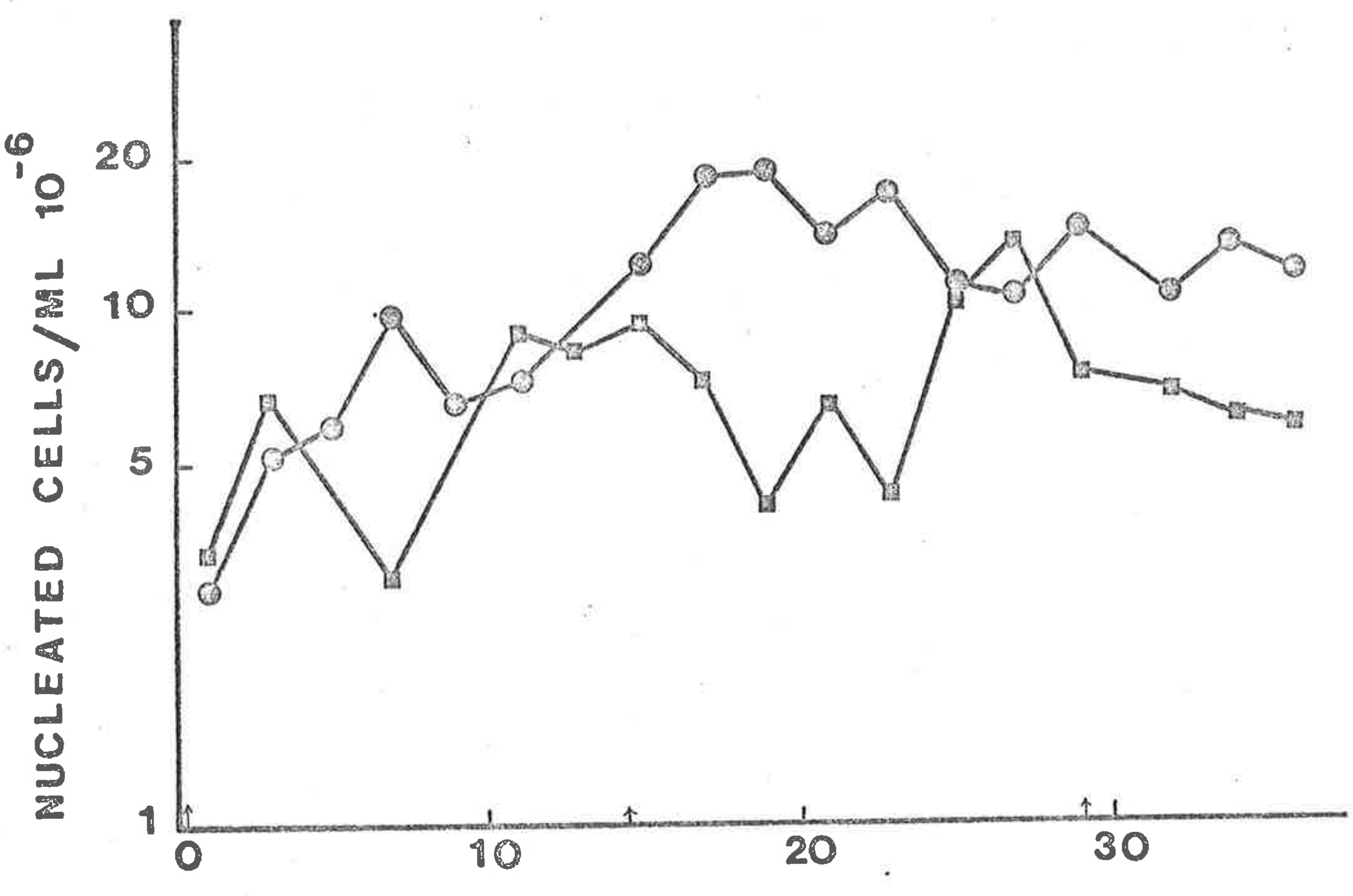

NUCLEATED CELLS/ML $10^{-6}$
20
10
5
1
0
10
20
30
TIME FOLLOWING INITIAL INFECTION DAYS

Table 5.3

The means and standard deviations of the total number of leukocytes in the peritoneal cavity following the intraperitoneal injections of <u>N. dubius</u> $L_3$

| Day following initial infection | Mean+ no. cells/ml ± S.D. ($\times 10^{-6}$) | |
|---|---|---|
| | Peritoneal exudate cells | Blood leukocytes |
| 0* | 1.5 ± 0.4 | 4.9 ± 0.5 |
| 3 | 5.2 ± 2.8 | 6.7 ± 2.4 |
| 5 | 6.0 ± 3.2 | 4.4 ± 1.6 |
| 7 | 9.9 ± 3.6 | 3.0 ± 0.9 |
| 9 | 6.7 ± 2.5 | 5.7 ± 1.2 |
| 11 | 7.2 ± 1.6 | 9.0 ± 1.8 |
| 13 | 9.5 ± 4.2 | 8.4 ± 3.6 |
| 15* | 12.3 ± 3.6 | 9.3 ± 2.4 |
| 17 | 18.1 ± 5.9 | 7.2 ± 1.8 |
| 19 | 18.2 ± 2.9 | 4.1 ± 0.9 |
| 21 | 13.4 ± 1.6 | 6.5 ± 1.3 |
| 23 | 17.1 ± 8.3 | 4.4 ± 0.7 |
| 25 | 10.9 ± 0.2 | 13.0 ± 4.4 |
| 27 | 10.6 ± 5.4 | 13.5 ± 7.5 |
| 29* | 14.4 ± 6.7 | 7.5 ± 2.2 |
| 32 | 11.0 ± 4.4 | 6.9 ± 1.5 |
| 34 | 13.6 ± 2.3 | 6.2 ± 1.4 |
| 36 | 11.8 ± 5.4 | 5.9 ± 0.9 |

\+ Arithmetic mean of 5 mice, same mice used as in Figure 5.7

* Administration of larvae.

Table 5.4

Eosinophils in the bone marrow of normal mice and mice following one oral infection by _N. dubius_ $L_3$.

| | % eosinophils+ in bone marrow |
|---|---|
| Uninfected mice | 1.5 |
| Infected mice* | 6.6 |

\+ Mean of 5 mice

* 13 days following a single infection with 200 _N. dubius_ $L_3$

that a primary infection did stimulate eosinophil production in the bone marrow but a secondary infection was needed for the appearance of these cells in the blood and peritoneal cavity.

6. Changes in serum immunoglobulin isotype levels following either multiple oral or intravenous administrations of $L_3$

Two groups of LACA mice were infected, one orally and the other intravenously with 200 $L_3$. On days 14 and 28 following the initial infection both groups received further doses of 200 $L_3$. Every 3-5 days 5 mice from each group were selected at random, bled and the serum pooled. The concentrations of IgM, IgA, $IgG_1$, $IgG_{2a}$ and $IgG_{2b}$ were determined by radioimmunassay in the serum of mice infected orally (Figure 5.8) and by the Mancini assay in the serum of mice infected intravenously (Figure 5.9).

There was an extraordinarily high, 20-100 fold increase in the levels of $IgG_1$, a concentration of 10 mg /ml being reached by day 17, 20 mg /ml by day 24-26 and in mice infected orally 30 mg /ml by day 31. This level was maintained for several weeks following the third infection. In contrast no consistent changes were observed in the concentrations of $IgG_{2a}$, $IgG_{2b}$ or IgA over the course of 3 infections. There were some fluctuations in the levels of $IgG_{2a}$ and $IgG_{2b}$ in the serum from mice infected intravenously. These are probably due to the relative inaccuracy of the Mancini assay when compared with the radioimmunoassay. The immunoglobulin levels in Figure 5.9 had been determined before the

Figure 5.8

Changes in serum immunoglobulin levels in mice infected orally with *N. dubius*. Animals were given 200 $L_3$ orally on days 0, 14 and 28 (↑).

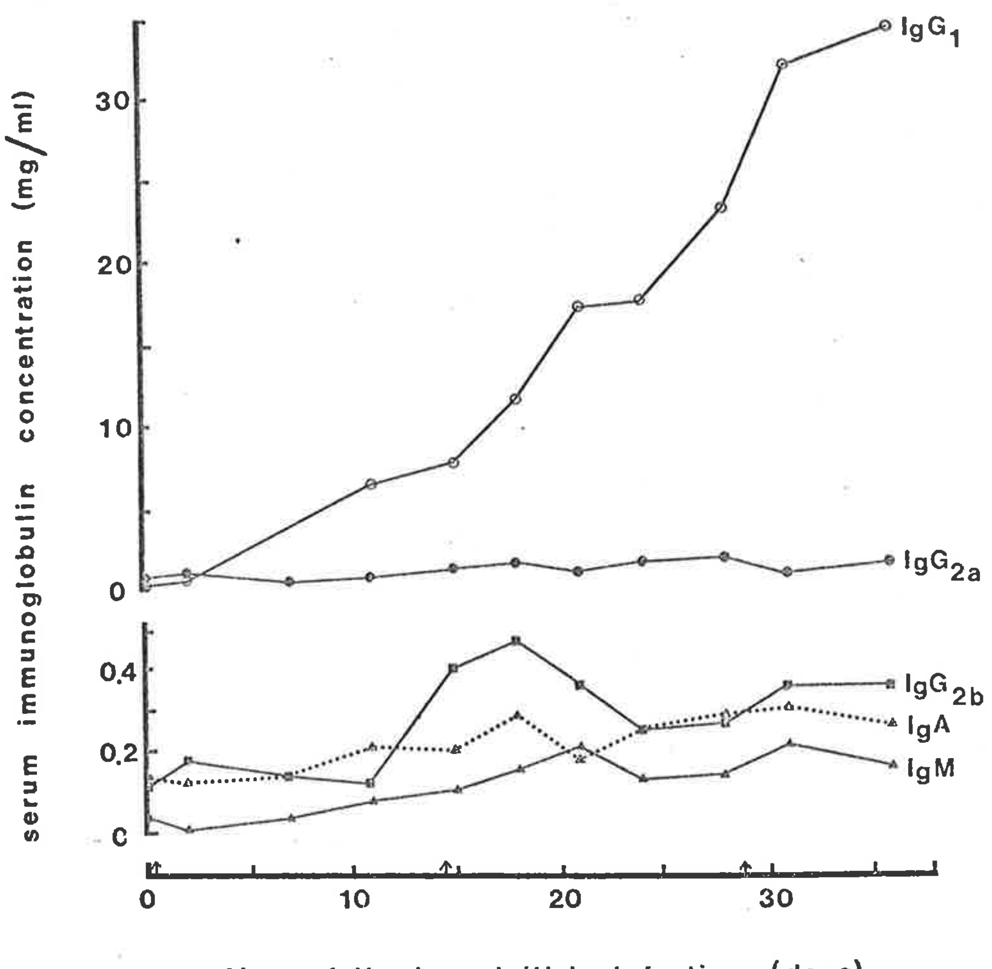

serum immunoglobulin concentration (mg/ml)
30
20
10
0
0.4
0.2
0
IgG1
IgG2a
IgG2b
IgA
IgM
0
10
20
30
time following initial infection (days)

Figure 5.9

Changes in serum immunoglobulin levels in mice injected intravenously with <u>N. dubius</u>. Animals were given 200 $L_3$ intravenously on days, 0, 14 and 28 (↑)

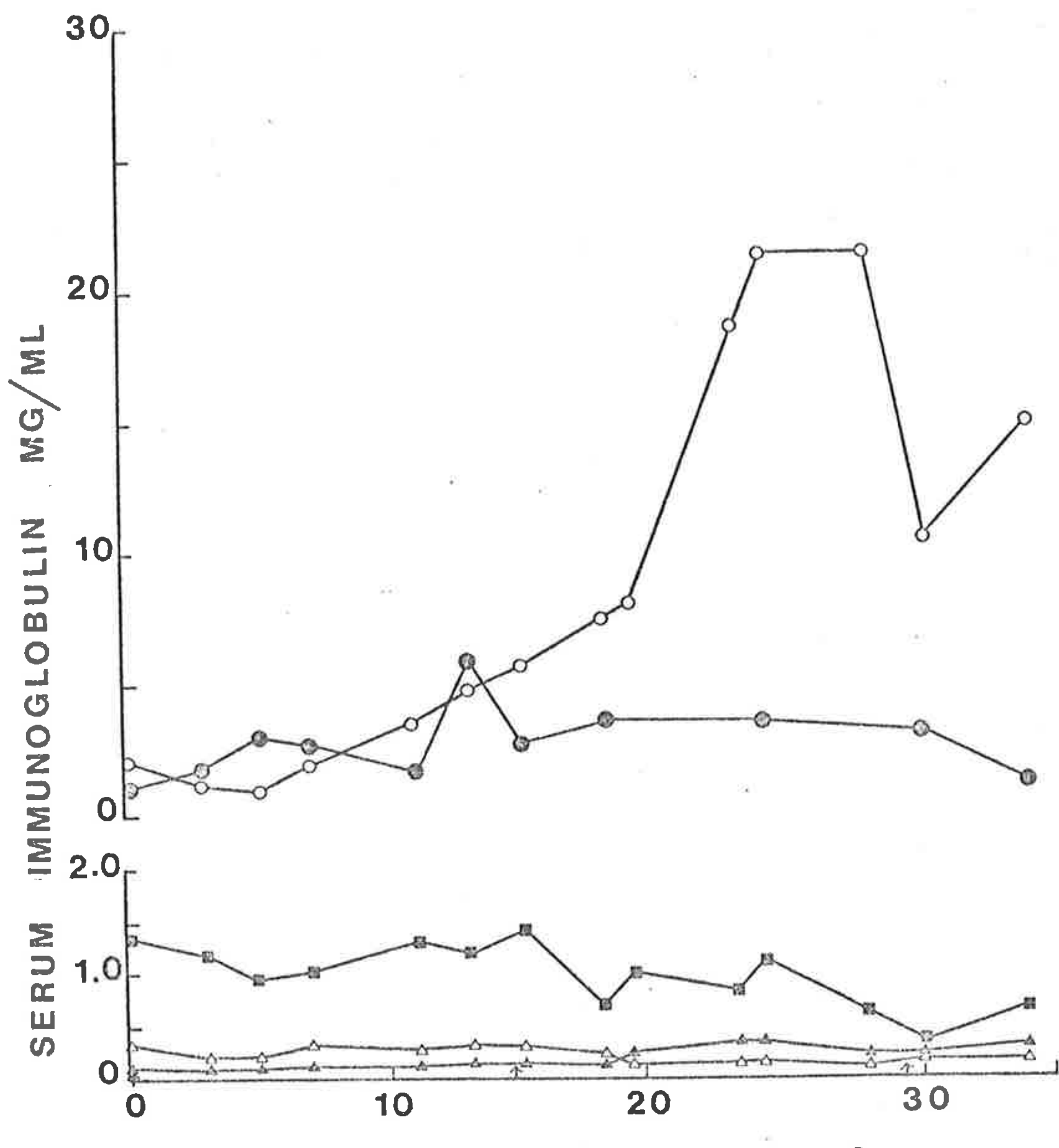
$IgG_1$ ○——○

$IgG_{2a}$ ●——●

$IgG_{2b}$ ■——■

IgM ▲——▲

IgA △——△



30
20
10
0
2.0
1.0
0
SERUM IMMUNOGLOBULIN MG/ML
0
10
20
30
TIME FOLLOWING INITIAL INFECTION DAYS

radioimmunoassay had been developed. There was a 5 fold increase in IgM, a plateau level of 0.15-0.2 mg /ml being attained by day 20.

7. Changes in serum $IgG_1$ and $IgG_{2a}$ concentration following a single infection of $L_3$

The immunoglobulin changes described above were the result of 3 infections of $L_3$. It was of interest to know whether a single infection would induce similar changes in the concentration of $IgG_1$ since mice that had received a primary infection were partially resistant to reinfection (See section 9). The concentration of the other major immunoglobulin isotype in mouse serum $IgG_{2a}$, was also measured.

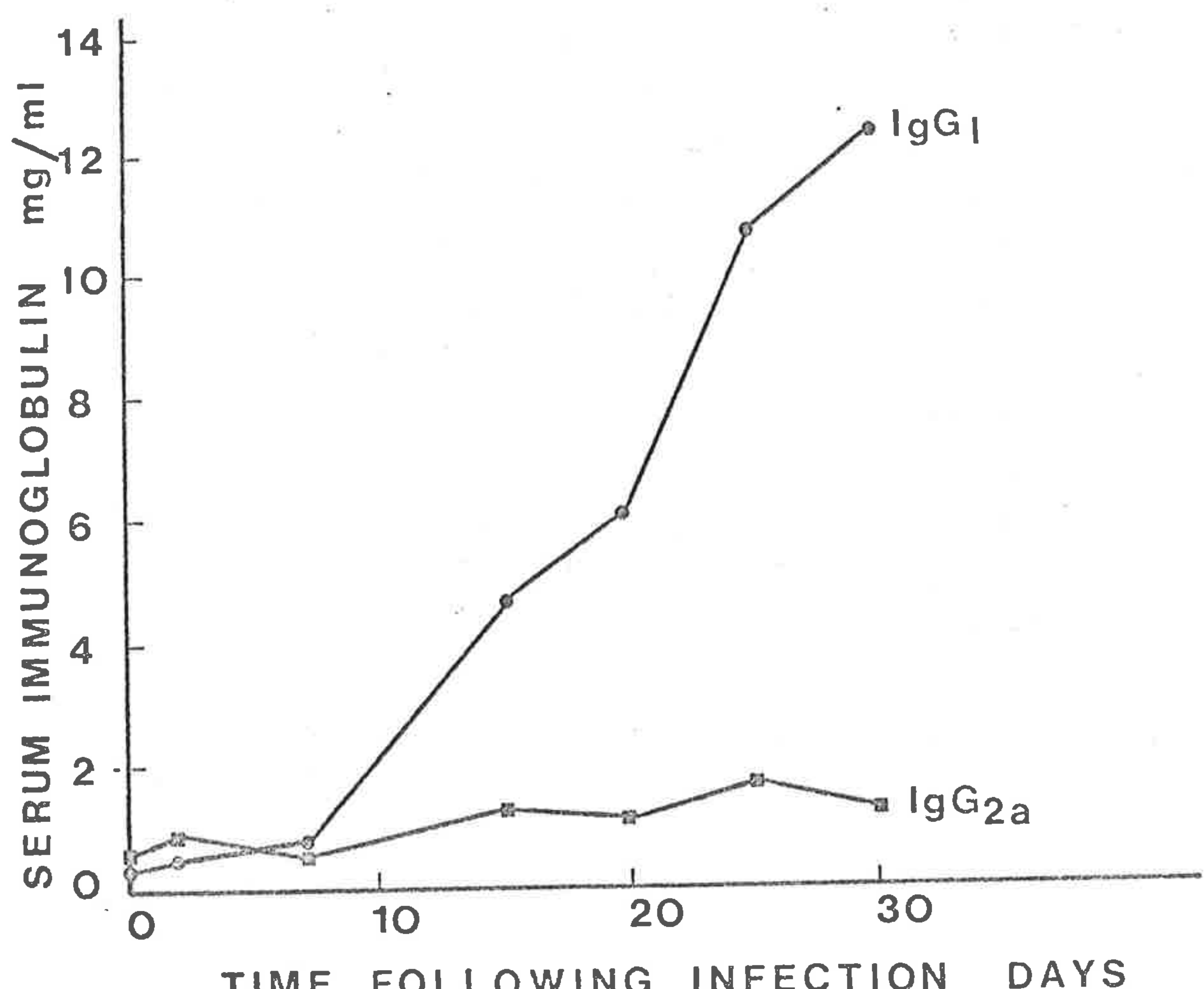
A group of LACA mice received one oral infection of 200 $L_3$. On days 0,2,7,15,20,25,30 and 35, 5 mice from the group were chosen at random and bled. The $IgG_1$ and $IgG_{2a}$ concentrations in the pooled serum was determined by radio-immunoassay. The results shown in Figure 5.10 indicate that even a single infection stimulates the production of remarkably high levels of $IgG_1$. In contrast to this the $IgG_{2a}$ levels remained constant throughout the period of measurement.

8. The role of adult worms in the stimulation of $IgG_1$ production

To investigate whether or not a particular stage in the life cycle of the parasite was responsible for the production of $IgG_1$, 2 groups of 10 mice were given 200 $L_3$

Figure 5.10

Changes in serum $IgG_1$ and $IgG_{2a}$ levels following a single oral infection of 200 *N. dubius* $L_3$.



SERUM IMMUNOGLOBULIN mg/ml
14
12
10
8
6
4
2
0
IgG1
IgG2a
0
10
20
30
TIME FOLLOWING INFECTION DAYS

orally on day 0 and again on day 26. One group, (A) was given pyrantel (12 mg/kg) orally on days 6,7,8 and again on days 31,32 and 33 to remove adult worms arising as a result of the second infection. The second group, (B) was not treated. The mice of both groups were bled on days 0, 26 and 40. An uninfected control group which had been kept in the animal house for the same length of time was also bled on day 40. The serum $IgG_1$ concentrations were determined on the serum pools by radioimmunoassay.

The $IgG_1$ concentration shown in Table 5.5 indicated that the adult worms may play a role in the initial stimulation of $IgG_1$ production. However the administration of a second dose of larvae caused the serum $IgG_1$ concentration of the group without adult worms, (A) to reach that of the group with adult worms (B) indicating that the larval stages can also stimulate $IgG_1$ production.

9. Time course for the development of resistance following intravenous and oral immunisation with third stage larvae

The above investigations have shown that infection by N. dubius induced marked changes in the total number of leukocytes in the blood and peritoneal cavity of the mouse, in the type of leukocytes present in the mouse and caused a spectacular increase in the serum concentration of one immunoglobulin isotype. The following experiments were carried out to determine if the development of resistance could be correlated with any of these changes.

Table 5:5

$IgG_1$ levels in serum pools from infected mice and from infected mice cleared of adult worms by antihelminth treatment.

| Time following infection, days | Serum $IgG_1$ + concentration (mg/ml) | |
|---|---|---|
| | Treated (A) | Untreated (B) |
| 0 | 0.25 | 0.25 |
| 26 | 2.9 | 9.6 |
| 40‡ | 16.6 | 17.8 |

\+ Determined by R.I.A.

‡ Uninfected mice kept under same conditions had serum $IgG_1$ concentration of 1.35 mg/ml on day 40.

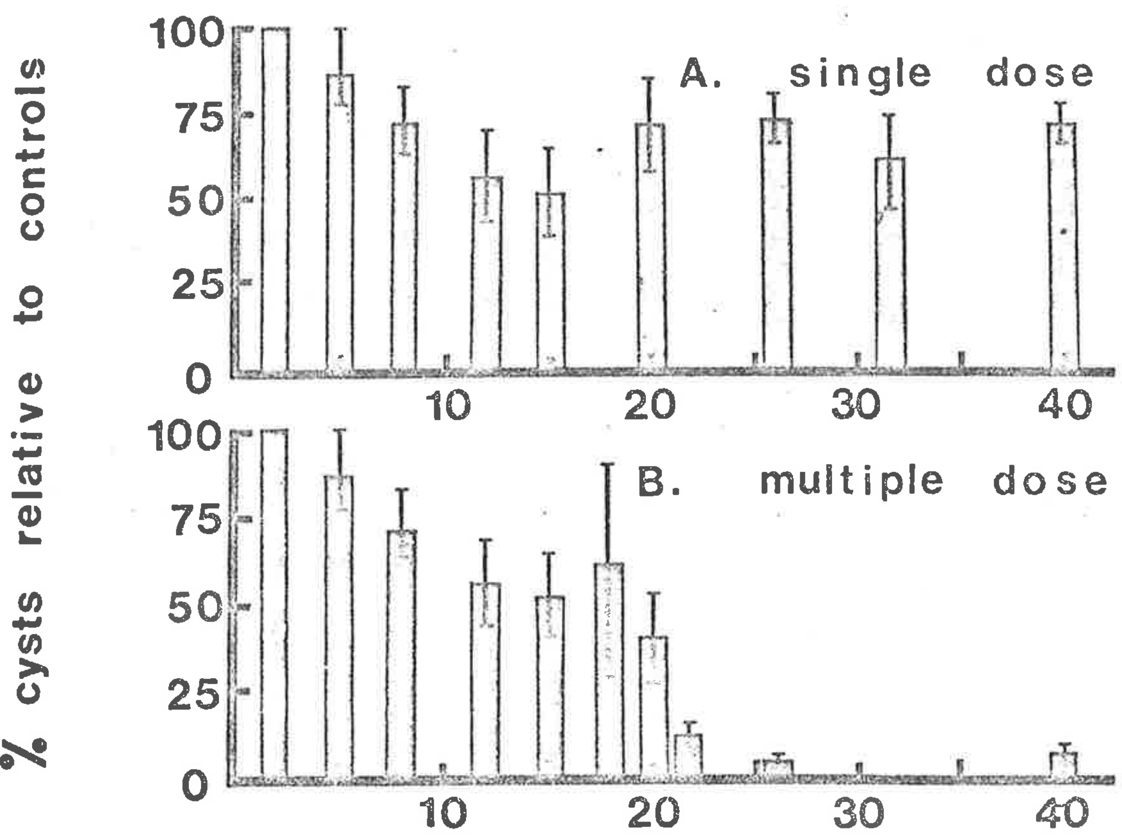
To determine how and when resistance developed a group of mice were given a single intravenous dose of $L_3$ and at chosen times there after groups of 6 animals picked at random and together with unimmunised control animals were challenged orally with 200 $L_3$ and the intestinal cysts counted 5 days later. The animals remaining in this group were given a second injection of larvae on day 14 and these were similarly challenged at selected times. The results, expressed as the number of cysts found in the immunised groups as a percentage of that observed in control animals are presented in Figure 5.11. A level of immunity in which 40-50% of the challenged dose of larvae are killed was induced within 8-10 days by a single immunising infection. Although this level of immunity was maintained for at least 5-6 weeks, it never increased beyond a capacity to kill about half of a challenge dose.

The effect of a second immunising infection can be seen in the lower part of figure 5.11. At the time of the second injection (day 14), the mice were able to kill only 50% of the challenge dose.

However the immunity of these animals rapidly matured such that within 8 days of the second dose, 90% or more of a challenge dose of larvae was killed. This immunity increased to a capacity for killing at least 95% of invading larvae, at which level it remained for approximately 2-3 months following a third immunising dose on day 28. The development of immunity in orally immunised mice is shown in Figure 5.12. Although done in less detail development of immunity followed

Figure 5.11

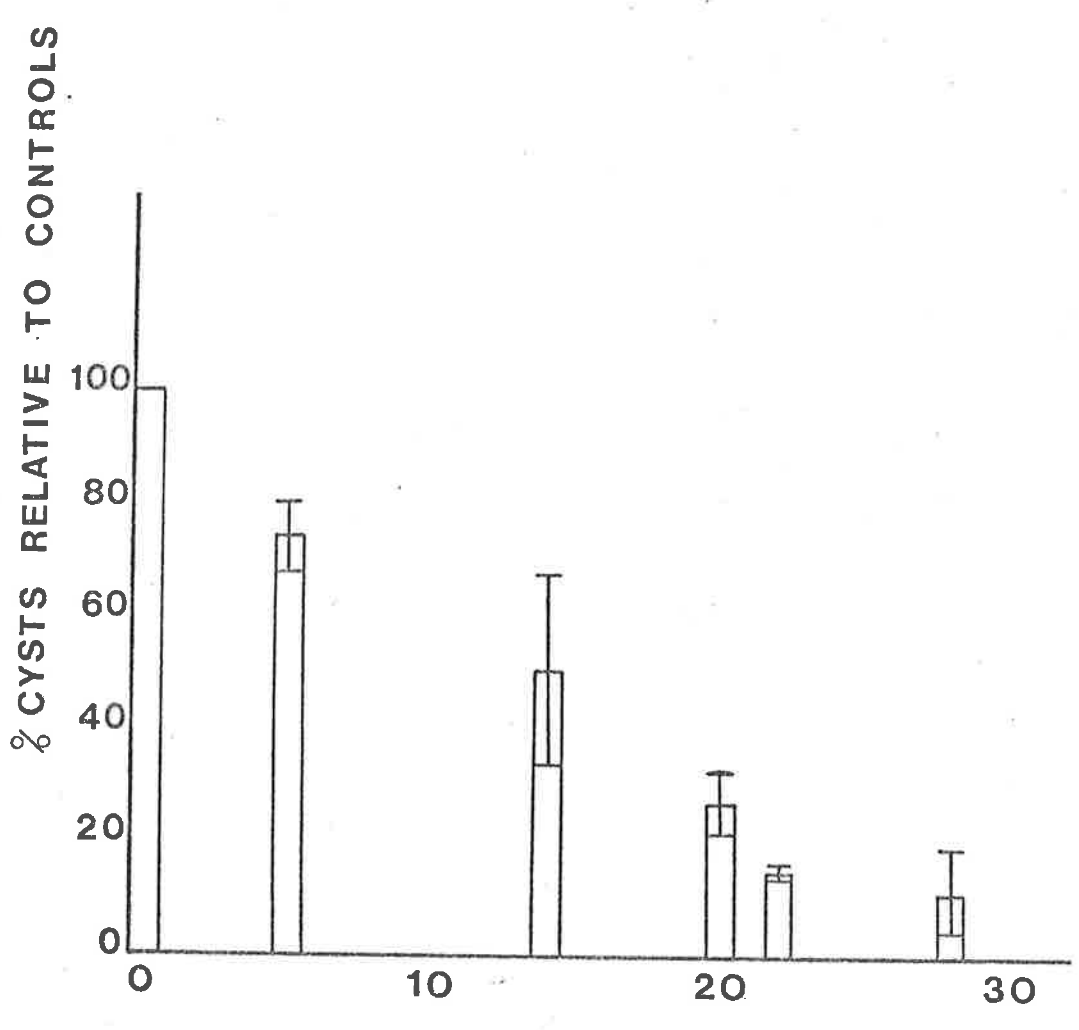
Time course of development of immunity to *N. dubius*. Mice were immunised intravenously with 200 third-stage larvae on day 0. At the times indicated, 6 mice were challenged orally with 200 larvae and cysts counted 5 days later. The lower figure (B) depicts the results for mice which were additionally immunised on days 14 and 28. Vertical bars represent 2 standard deviations.



A. single dose
B. multiple dose
100
75
50
25
0
10
20
30
40
% cysts relative to controls
time following initial infection (days)

Figure 5.12

Time course of the development of immunity to *N. dubius*. Mice were immunised orally with 200 $L_3$ on day 0, at times indicated, 6 mice were challenged orally with 200 $L_3$ and cysts counted 5 days later. Vertical bars represent 2 standard errors.


% CYSTS RELATIVE TO CONTROLS
100
80
60
40
20
0
0
10
20
30
TIME FOLLOWING INITIAL INFECTION DAYS

essentially the same pattern as in mice immunised intravenously.

10. Enhanced protection of infected mice following the administration of serum from immune mice

Details of the biological activities of mouse $IgG_1$ have not been clearly elucidated. However $IgG_1$ is reputedly unable to fix complement by the classical pathway or bind to normal macrophages, (Nussenzweig, Merryman and Benacerraf, 1964; Spiegelberg, 1974) although firm evidence in these points is still lacking.

Since infection of mice by N. dubius induced such high levels of serum $IgG_1$ it was of interest to determine what possible function this isotype may play in the expression of resistance. One possible way to study this was to transfer serum from immune mice to mice which had received a single infection of N. dubius $L_3$. If antibody of the $IgG_1$ isotype directed against parasite antigens played no role in protection it may have been expected to block the expression of resistance against the parasite by binding to parasite antigens and preventing contact between the parasite and sensitised cells and/or antibodies of different isotypes. However, if the $IgG_1$ played an active role in protection, the transfer of serum from immune mice or more importantly $IgG_1$ from this serum, should enhance resistance.

In the first experiment 3 groups of 7 mice were used. The first group, (A) was a control group which received only the challenge dose of 200 $L_3$ orally. The other 2 groups were used on day 13 following a single dose of 200 $L_3$ and

received either serum from mice immune to reinfection (B) or normal mouse serum (C). They received 1 ml of serum intraperitoneally on day 13, which was also the day they were challenged together with the control (A) mice. Further doses of 0.75 ml of serum were given on days 14 and 15 and the cysts counted in all groups 5 days following the challenge. At the time of cyst counting the level of peritoneal eosinophilia was also determined in the control group and the group which had received serum from immune mice.

The results in Table 5.6 show that the administration of serum from immune mice to mice which had received one previous infection greatly enhanced the degree of protection obtained when compared to those mice which received normal mouse serum. It was interesting to note that the mice which received immune mouse serum also had a substantial number of eosinophils at the time of cyst counting.

The second experiment was carried out in order to ascertain the importance of antibody of the $IgG_1$ isotype in mediating this effect. In this experiment one group of 5 naive mice (A) together with 4 groups of 5 mice (B-E) which had undergone one infection 13 days previously, were used. The second group (B) was given $IgG_1$ isolated from the serum of mice immune to reinfection. The $IgG_1$ (1 mg/ml) was injected intraperitoneally at doses of 2.5 ml on day 13 and 14 and 1 ml on day 15. The third group, C received intra-peritoneally, 1 ml of serum from immune mice on days 13 and 14 and 0.75 ml on day 15. Group D was injected intraperitoneally with 2 ml of effluent from the protein A - sepharose column

Table 5.6

Enhanced protection of mice by the administration of, serum or serum fractions from resistant or normal mice to mice following a single immunising infection with $L_3$

| Group | Treatment | No. mice | Cysts(day 18)+ | % peritoneal eosinophils |
|---|---|---|---|---|
| Expt. 1 | | | | |
| A | none | 7 | 178 ± 2 | 0 |
| B | 200 $L_3$* normal mouse serum | 7 | 80 ± 2 | N.D. |
| C | 200 $L_3$ immune mouse serum | 7 | 20 ± 2 | 12.6 |
| Expt. 2 | | | | |
| A | none | 5 | 194 ± 8 | 0 |
| B | 200 $L_3$ ‡ $IgG_1$ | 5 | 67 ± 11 | 9.4 |
| C | 200 $L_3$ immune mouse serum | 5 | 52 ± 17 | N.D. |
| D | 200 $L_3$# effluent, Protein A | 5 | 127 ± 7 | 7 |
| E | 200 $L_3$ saline | 5 | 117 ± 17 | 4 |

\+ Arithmetic mean ± S.E.

* mice used on day 13 following infection

‡ $IgG_1$ from immune mouse serum, isolated from protein A-sepharose

\# Contained 2.5 mg/ml protein, no IgG.

(2.5 mg/ml protein) at a dose of 2 ml on days 13 and 14 and 1 ml on day 15. This contained no IgG. Finally group E received injections of saline at the same dose schedule as immune mouse serum, 1 ml on days 13 and 14 and 0.75 ml on day 15. All mice together with the control group (A) were challenged orally on day 13 and the cysts counted on day 18. Peritoneal eosinophil counts were carried out on day 18 before cyst counting.

The results in Table 5.6 show that both immune mouse serum and $IgG_1$ from immune mouse serum enhanced protection in mice following a single infection of N. dubius. Since the effect was not mediated by the effluent from the protein A-sepharose column nor by normal mouse serum or saline it seemed that at this stage in the development of resistance the presence of antibody in the $IgG_1$ isotype is a factor limiting the expression of resistance. All the mice which had been infected once had eosinophils in the peritoneal cavity when tested but there was no indication as to whether these resulted from the injection of material into the peritoneal cavity, the second (challenge) infection and/or result from the killing of some of the worms in the intestinal wall.

11. Serum $IgG_1$ and $IgG_{2a}$ concentrations in infected mice of inbred strains

The production of extremely high levels of $IgG_1$ immunoglobulin has been shown above to be a characteristic of N. dubius infections. In order to determine whether differences in resistance and susceptibility of the inbred strains of mice (Chapter 3) were reflected in the levels

of $IgG_1$, serum samples were taken from the immunised mice (Table 3.3 Chapter 3), at the time of cyst counting and the $IgG_1$ levels determined on serum pools by radioimmunoassay (RIA). In addition, levels of the other major serum immunoglobulin isotype, $IgG_{2a}$ were determined. The $IgG_1$ and $IgG_{2a}$ levels in the isogenic strains are shown in Table 5.7. All the strains studied, both males and females, produced high levels of $IgG_1$ (range, 18-46 mg/ml) similar to those described by Crandall, Crandall and Franco, (1974). $IgG_{2a}$ levels in all strains were relatively low, similar to those concentrations reported above. There was no apparent correlation between the degree of resistance and serum $IgG_1$ and $IgG_{2a}$ levels.

12. Conclusions

The study of the cell and immunoglobulin changes during infection indicated that multiple infections had wide ranging effects on peripheral and peritoneal leukocyte levels and immunoglobulin production. There were changes in the number of macrophages, lymphocytes, neutrophils and monocytes. However the most dramatic changes in resistance occurred with the appearance of eosinophils. A single infection did not result in eosinophils appearing peripherally although they were present in increased numbers in the bone marrow. A further stimulation resulted in the appearance of eosinophils in the blood and peritoneal cavity.

The immune mice also had remarkably high levels of serum $IgG_1$. A single infection primed the animals for the maximum production of $IgG_1$ which occurred following the 2nd

Table 5.7

Serum $IgG_1$ and $IgG_{2a}$ concentrations in immunised S.P.F. derived mice*.

| Mouse strain | C57Bl/6 | DBA/2 | BALB/c | C3H/HeJ | CBA/H | SJL/J | A/J |
|---|---|---|---|---|---|---|---|
| Sex+ | F | F | F | F | M | F | M |
| $IgG_1$ (mg/ml) | 45.8 | 32.0 | 30.2 | 24.5 | 18.5 | 21.3 | 32.3 |
| $IgG_{2a}$ (mg/ml) | 0.18 | 0.19 | 0.02 | 2.3 | 0.71 | 0.18 | 1.0 |

* The pre-infected (immunised) mice used in Table 3.3 were bled at day 5 (after oral $L_3$ administration on days -22, -10, and 0) and IgG levels determined on pooled serum samples by RIA.

\+ Abbreviations F = female, M = male.

administration of larvae. The peak of the $IgG_1$ levels and the appearance of eosinophils correlated with the development of full protection following the 2nd dose of larvae (days 20-22). Enhanced protection in animals which had received a single dose of $L_3$ could also be elicited by the administration of serum or $IgG_1$ from immune mice. The ability of $IgG_1$ to enhance protection indicated that there may be antibody in this isotype directed against parasite antigens and this may play a role in the expression of resistance.

These results also demonstrated that there were no differences between the effects of oral or intravenous administration of larvae.

# CHAPTER 6

## *N. dubius* infection in congenitally athymic (nude) mice

1. Introduction
2. Comparison of the susceptibility of nu/nu and nu/+ mice to *N. dubius* infection
3. The development of resistance in nu/nu, nu/+ and nu/nu mice injected with syngeneic lymphoid cells.
4. The effect of the transfer of serum from hyperimmune intact mice to naive nude mice
5. Immunoglobulin levels of infected nu/nu, nu/+ and nu/nu mice given syngeneic lymphoid cells
6. The effect of the degree of reconstitution on serum immunoglobulin levels in infected nu/nu mice
7. Conclusions

## 1. Introduction

The experiments described in Chapter 5 showed that there was a strong correlation between the onset of good protection, the appearance of eosinophils in the blood and peritoneal cavity of immunised mice and the peak of the $IgG_1$ serum immunoglobulin level.

Previous studies (Chaicumpa and Jenkin, 1977) have suggested that activated macrophages may play a role in protection. In order to determine the role in resistance to infection of responses such as eosinophilia and $IgG_1$ production, which are dependent upon thymus derived lymphocyte (T cell) function, the development of resistance and changes in immunoglobulin levels in athymic (nude) mice were studied. Although nude mice have greatly reduced T cell dependent responses they are reported to have activated macrophages (Cheers and Waller, 1975). Thus the nude mouse would apparently be a useful model to dissect further, the relative importance of the above changes seen in the intact mouse, in the development of protective immunity.

## 2. Comparison of the susceptibility of nu/nu and nu/+ mice to N. dubius infection

Female nu/nu and nu/+ mice were dosed orally with 200 or 250 $L_3$. Five days after infection, some of the mice were killed, the intestines removed and the number of cysts counted. Granulomata, adult worms and residual

cysts were counted in the remainder of the mice on day 10. The results in Table 6.1 show that equal numbers of encysted larvae and adult worms were found in the nu/nu and the nu/+ animals. This indicates that establishment of a first infection proceeds independently of the T cell status of the host. In contrast, the granulomata counts show that the nu/nu mice lacked the ability to form the granulomatous lesions which develop in normal mice at the vacated site of larval encystment in the intestinal wall. Granulomata development therefore appears to be highly T cell-dependent as shown by Bartlett and Ball,(1974).

3. The development of resistance in nu/nu, nu/+ and nu/nu mice injected with syngeneic lymphoid cells.

Since nu/nu mice and nu/+ mice were equally susceptible to a primary infection several experiments were carried out investigating the ability of nu/nu, nu/+ and nu/nu mice injected with syngeneic lymphoid tissue to develop resistance. Male or female mice were used as donors of the lymphoid tissue in separate experiments since it was demonstrated previously that female mice developed better resistance than male mice (Chapter 3).

In the initial experiment, 4 female nu/nu mice, 7 female nu/+ mice and 7 female nu/nu mice injected with syngeneic lymphoid cells as indicated in Table 6.2 received 200 $L_3$ intravenously on days -20 and -10 and were challenged orally together with 5 nu/nu and 5 nu/+ as control mice, with 100 $L_3$ on day 0.

Table 6.1

The number of granulomata, cysts and adult worms in nu/nu and intact nu/+ mice following a primary infection with *N. dubius*

| BALB/c mice | | | Number of administered $L_3$ | Day when mice killed | Infection Status | | |
|---|---|---|---|---|---|---|---|
| Type | Age at day 0 (weeks) | No. | | | No. of cysts* | No. of adults* | No. of Granulomata* |
| nu/nu | 6 | 4 | 250 | 5 | 218±15 | - | - |
| | | 5 | 200 | 5 | 184±25 | - | - |
| | | 4 | 250 | 10 | 14±1 | 194±4 | <2 |
| nu/+ | 6 | 4 | 250 | 5 | 211±5 | - | - |
| | | 5 | 200 | 5 | 184±28 | - | - |
| | | 4 | 250 | 10 | 9±2 | 200±6 | 171±5 |

* Arithmetic mean ± S.E.

The results are shown in Table 6.2. The nu/+ mice developed a high level of protection as indicated by the reduction in the number of cysts in the immunised nu/+ mice when compared with the control group. The nu/nu mice also developed a degree of protection as indicated by the reduction in cyst numbers when compared with the controls. Surprisingly the mice injected with cells were not significantly more protected than the nu/nu mice.

In order to determine the fate of adult worms arising from the immunising infections and the ability of immunised nu/nu mice to form granulomata, two further experiments were carried out. Since the cell injected mice used in the previous experiment did not develop good resistance the number of lymphoid cells injected into the nu/nu mice was increased. In these experiments groups of female nu/nu mice, their heterozygous littermates and nu/nu mice which were injected with pooled thymus and mesenteric lymph node cells from male BALB/c nu/+ and +/+ donors (Expt.1) or female BALB/c nu/+ donors (Expt.2) at a ratio of 5 donors to one recipient were used. These mice were infected orally with $L_3$ as indicated in Table 6.3. The groups of mice to be immunised received three administrations of larvae 12 days apart with the final administration being the challenge dose. Five days after the challenge, the number of encysted larvae was determined, as was the number of granulomata and adult worms arising from the immunising doses. Control mice were unimmunised age- and sex-matched

Table 6.2

The development of immunity to <u>N. dubius</u> in nu/+, nu/nu or nu/nu mice given syngeneic lymphoid cells

| BALB/c mice# | | Administrations of $L_3$ | No. cysts* day 5 |
|---|---|---|---|
| Type | No. | | |
| nu/nu | 5 | 1 | 102 ± 7 |
| | 4 | 3 | 58 ± 8 |
| nu/+ | 5 | 1 | 100 ± 4 |
| | 7 | 3 | 9 ± 2 |
| nu/nu plus cells‡ | 7 | 3 | 54 ± 2 |

# Mice aged 6 weeks on day -20

* Arithmetic mean ± S.E.

‡ Injected intraperitoneally with pooled mesenteric lymph node and thymus cells from male nu/+ mice at a ratio of 3 donors per recipient.

animals which had received only the challenge dose. The results in Table 6.3 show that nu/nu mice can develop a degree of protection against a challenge infection. This is indicated by a 33 to 50% reduction in cyst numbers in the immunised nu/nu mice in both experiments. The nu/+ animals were almost fully protected against the challenge infection with a 90-95% reduction in the number of cysts when compared with control animals given a single dose of $L_3$. However the cell injected nu/nu mice were only marginally more protected than the nu/nu mice in terms of cyst numbers (experiment 1, $0.001<P<0.002$; expt. 2, $0.02<P<0.05$) irrespective of whether male or female mice were used as lymphoid cell donors.

The number of adult worms found in nu/nu mice indicated that worms resulting from the immunising infections accumulated and were not lost. In contrast, the immunised nu/+ mice seemed to have eliminated substantial numbers of their adult worms, almost all being lost in experiment 1 and a smaller but still significant ($P<0.001$) loss occurring in experiment 2. The cell-injected nu/nu mice, however, had adult worm burdens only marginally less than those of the nu/nu mice (expts. 1 and 2, $0.01<P<0.02$). It therefore seems that injection of nu/nu mice with cells from male (expt. 1) or female (expt. 2) donors did not fully restore the capacity of the host to eliminate adult worms. The granulomata numbers indicated that the nu/nu mice were unable to form granulomata even after two immunising infections. Cell-injected nu/nu mice were able to form

Table 6.3

The development of resistance to N. dubius and the fate of adult worms in nu/+, nu/nu or nu/nu mice given syngeneic lymphoid cells

| Expt. | BALB/c mice | | Naive mice* (controls) | | Preinfected (immunised) mice[‡] | | | |
|---|---|---|---|---|---|---|---|---|
| | Type | Age (weeks) at day -24 | No. | No. cysts day 5** | No. | No. cysts day 5** | No. adult worms** | No. granulomata** |
| 1 | nu/nu | 7 | 6 | 163 ± 5 | 10 | 81 ± 7 | 282 ± 14 | < 3 |
| | nu/+ | 7 | 6 | 142 ± 11 | 9 | 7 ± 1 | 4 ± 1 | 117 ± 9[#] |
| | nu/nu plus cells (M)§ | 7 | 6 | 177 ± 10 | 9 | 48 ± 4 | 232 ± 11 | 44 ± 10 |
| 2 | nu/nu | 8 | 5 | 206 ± 8 | 7 | 138 ± 12 | 296 ± 4 | 0 |
| | nu/+ | 8 | 5 | 217 ± 8 | 7 | 11 ± 3 | 91 ± 17 | 124 ± 6[#] |
| | nu/nu plus cells (F)§ | 8 | 5 | 215 ± 11 | 6 | 101 ± 11 | 251 ± 12 | 6 ± 2 |

* Received 200 $L_3$ (Expt.1) or 250 $L_3$ (Expt.2) orally on day 0

‡ Received 200 $L_3$ orally on days -24, -12 and 200 $L_3$ (Expt.1) or 250 $L_3$ (Expt.2) orally on day 0.

** Arithmetic mean ± standard error

\# Granulomata noticeably more intense in nu/+ than in cell injected nu/nu mice.

§ Mice injected with pooled thymus and mesenteric lymph node cells from BALB/c males (nu/+ and +/+) in a ratio of 5 donors per recipient (Expt.1) and from female BALB/c nu/+ mice in a ratio of 5 donors per recipient (Expt.2).

granulomata but these were fewer in number than in nu/+ mice and were noticeably less intense. The infiltration of cells to intestinal wall lesions and thus granuloma formation, was markedly defective in mice lacking a normal complement of T cells.

4. The effect of the transfer of serum from hyperimmune intact mice to naive nude mice

Many attempts have been made to transfer protection against *N. dubius* using serum from resistant mice,(Panter, 1969b; Cypess, 1970; Chaicumpa, Jenkin and Rowley, 1976). These have all been unsuccessful and have led to the idea that a change in the activity of phagocytic cells and/or the appearance of a different type of cell is involved in immunity (Chaicumpa and Jenkin, 1978).

It is known that nude mice have activated macrophages and their high initial resistance to intracellular parasites such as *Salmonella typhimurium* and *Listeria monocytogenes* has been attributed to this state of activation (Cheers and Waller, 1975; Nichol and Bonventre, 1977). It seemed possible that the degree of protection obtained in nude mice may be due to the action of these activated macrophages and a T independent antibody response. If this were so then a degree of protection should be obtained in nu/nu mice by the transfer of serum from intact mice, resistant to infection, similar to that observed by the active immunisation of nu/nu mice with $L_3$.

To test this proposal, 2 groups of 6-8 week old female BALB/c nu/nu mice were given 200 $L_3$ orally. One

group received serum from immune mice and the second group, serum from normal mice. Both groups received 0.5 ml of serum intraperitoneally on day 0, i.e. the day of challenge and days 1,2, and 3 post challenge. Cysts were counted on day 5.

The data in Table 6.4 indicates that the group which had received serum from immune mice was significantly protected from infection when compared with the mice which had received normal mouse serum. This suggested that antibody in the serum from immune mice may be important in mediating the killing of $L_3$ in vivo, presumably by directing the activated macrophages to the site of larval penetration of the intestinal wall.

5. Immunoglobulin levels of infected nu/nu, nu/+ and nu/nu mice given syngeneic lymphoid cells

In order to study further the differences in the responses of the nu/nu, nu/+ and the cell-injected nu/nu mice, immunoglobulin determinations were carried out on serum pools from the immunised animals in experiment 1 (Table 6.3). The changes in serum immunoglobulin levels over the course of three infections can be seen in Figure 6.1. The maximum concentrations of $IgG_1$, $IgG_{2a}$ and IgM observed in a similar experiment are given in Table 6.5. The infected nu/nu mice had low concentrations (<3.5 mg/ml) of $IgG_1$, $IgG_{2a}$ and IgM. Although the $IgG_1$ concentration in the infected nu/+ mice reached extraordinarily high levels (20-50 mg/ml), there was no change in the $IgG_{2a}$ level, which remained even lower than that detected in

Table 6.4

The effect of transfer of serum from intact mice resistant to reinfection by N. dubius or normal mice to challenged+ BALB/c nu/nu mice

| Serum transferred‡ | no. mice | day 5 cysts |
|---|---|---|
| normal | 6 | 181 ± 6# |
| immune* | 6 | 106 ± 11§ |

+ All mice received 200 $L_3$ orally on day 0.

‡ mice received 0.5 ml of serum IP on days 0,1,2, and 3

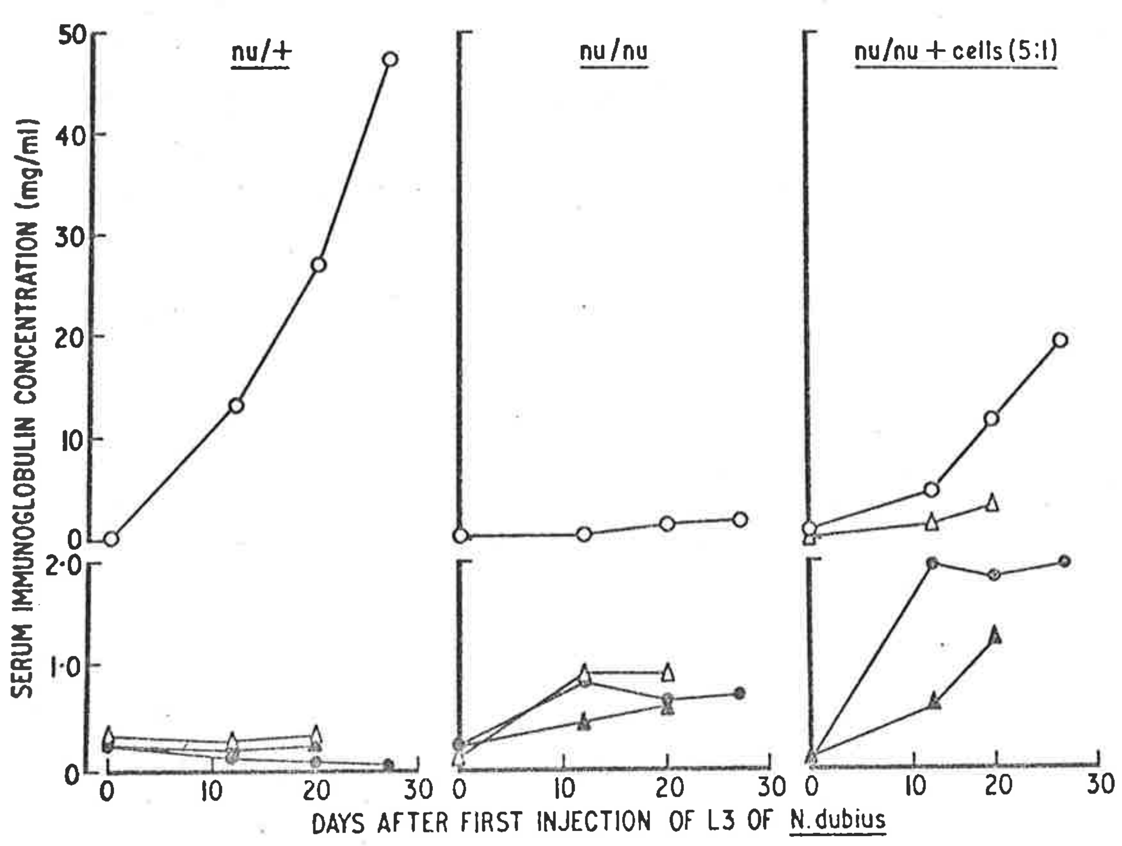
* serum from mice resistant to reinfection by N. dubius

# Arithmetic mean ± S.E.

§ Statistical analysis 'U' test of Mann and Whitney P=0.001.

Figure 6.1

Levels of serum $IgG_1$ (open symbols) and $IgG_{2a}$ (closed symbols) determined by radioimmunoassay in pools of sera from SPF-derived female BALB/c mice (nu/+, nu/nu or nu/nu injected with a mixture of thymus and mesenteric lymph node cells from male BALB/c-nu/+ mice at the ratio of 5 donors per recipient). Each group of mice was either uninfected (triangles) or given third stage larvae of N. dubius orally at doses of 200 on day 0, 200 on day 12 and 150 on day 22 (circles), (See expt. 1, Table 6.3).



nu/+
nu/nu
nu/nu + cells (5:1)
SERUM IMMUNOGLOBULIN CONCENTRATION (mg/ml)
50
40
30
20
10
0
2·0
1·0
0
0
10
20
30
DAYS AFTER FIRST INJECTION OF L3 OF N. dubius

Table 6.5

Maximum $IgG_1$, $IgG_{2a}$ and IgM concentrations in pooled sera from SPF-derived female mice bled at various times over a 30 day period in which $L_3$ of N. dubius were given 3 times (See Table 6.2)

| Mice | Immunoglobulin conc. mg/ml+ | | |
|---|---|---|---|
| | $IgG_1$ | $IgG_{2a}$ | IgM |
| BALB/c-nu/nu | 2.3 | 3.2 | 0.6 |
| BALB/c-nu/+ | 17.5 | 1.2 | 1.1 |
| BALB/c-nu/nu given thymus cells* | 3.2 | 8.8 | 1.1 |

\+ Determined by radial immunodiffusion

* Thymus and mesenteric lymph node cells from male BALB/c-nu/+ and +/+ were given at a dose of 3 donors per recipient (See Table 6.2)

nu/nu mice. The cell-injected nu/nu mice had higher levels of $IgG_1$ than the nu/nu mice but much less than the nu/+ animals. The reconstituted mice, however had a high level of $IgG_{2a}$ (8.8 mg/ml, Table 6.5; 2 mg/ml Fig. 6.1). This indicated that $IgG_1$ hypergammaglobulonemia was highly T cell-dependent and suggested that T cell-injected nu/nu mice did not have the numbers of T cells which were required to completely redirect the response into $IgG_1$ production. The levels of serum $IgG_{2b}$ and IgA did not change over the course of multiple infections.

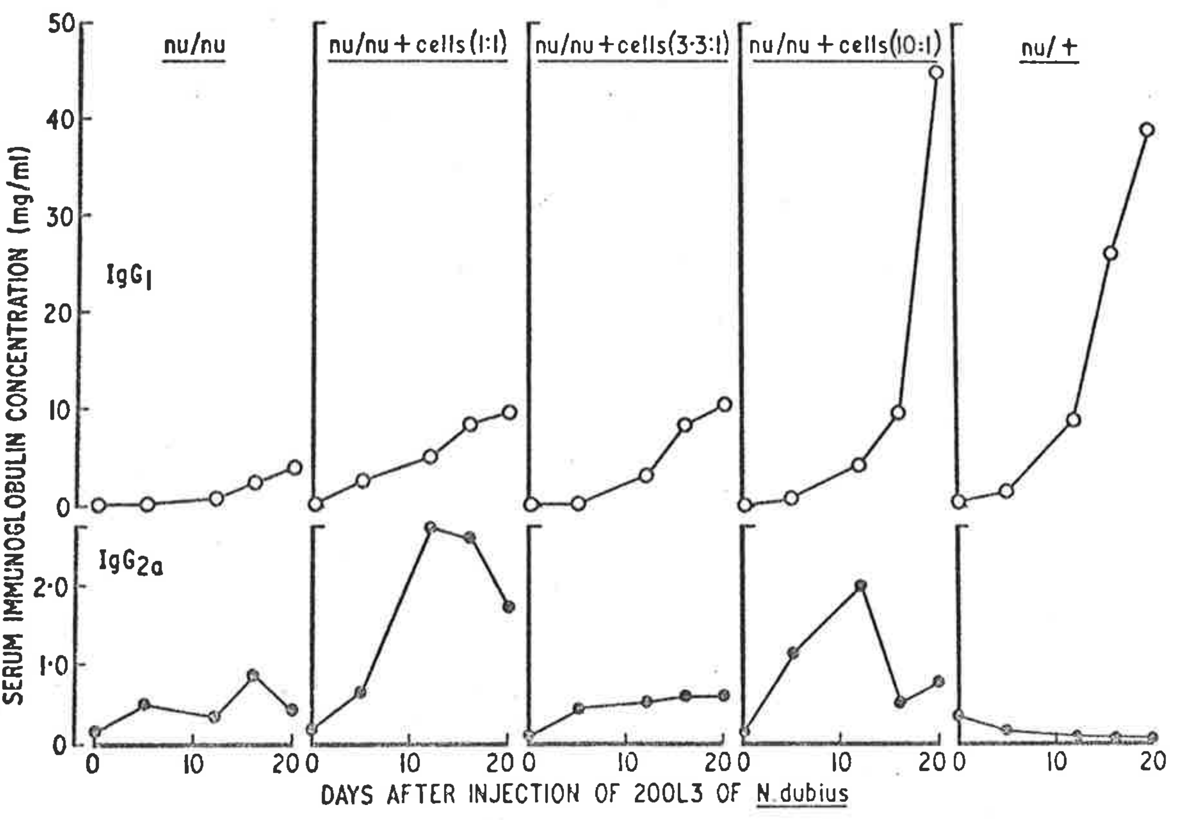
6. The effect of the degree of reconstitution on serum immunoglobulin levels in infected nu/nu mice

In order to investigate the effect on immunoglobulin levels of the number of T cells used for reconstitution, 3 groups of BALB/c nu/nu mice were injected with syngeneic cells in ratios of one donor per recipient (1:1), 3.3 donors per recipient (3.3:1) or 10 donors per recipient (10:1) using cortisone-resistant thymocytes as described in Materials and Methods. These mice, together with 6 nu/nu and 6 nu/+ mice, were infected with 200 $L_3$ of N. dubius. They were bled on days 0,5,12,16 and 20 and the serum immunoglobulin concentrations determined (Figure 6.2).

The nu/+ mice had the lowest levels of $IgG_{2a}$ suggesting that infected animals with a full complement of T cells synthesise virtually no $IgG_{2a}$ or that the catabolic rate of $IgG_{2a}$ is increased. The highest $IgG_{2a}$ level was found in nu/nu mice injected at a cell ratio of one donor per recipient. As the degree of reconstitution increased,

Figure 6.2

Levels of serum $IgG_1$ (open symbols) and $IgG_{2a}$ (closed symbols) determined by radio-immunoassay in pools of sera from SPF-derived female BALB/c mice (nu/+, nu/nu or nu/nu injected with thymus cells from BALB/c-nu/+ males given 1.75 mg of hydrocortisone 2 days previously at ratios of 1, 3.3 or 10 donors per recipient). Each group of mice was given an oral dose of 200 $L_3$ of *N. dubius* on day 0.


nu/nu
nu/nu + cells (1:1)
nu/nu + cells (3·3:1)
nu/nu + cells (10:1)
nu/+
SERUM IMMUNOGLOBULIN CONCENTRATION (mg/ml)
IgG1
IgG2a
50
40
30
20
10
0
2·0
1·0
0
0 10 20
DAYS AFTER INJECTION OF 200L3 OF N. dubius

immunoglobulin synthesis seemed to be increasingly directed into $IgG_1$ synthesis. The infected heterozygotes and nu/nu mice reconstituted at a ratio of 10:1 had similar levels of $IgG_1$ (40-50 mg/ml). However these reconstituted nu/nu mice still produced $IgG_{2a}$ indicating that even this high level of T cell reconstitution was not sufficient to reduce $IgG_{2a}$ levels to those observed in infected nu/+ mice.

7. Conclusions

These results show that nu/nu mice following two infections may become partially resistant to rechallenge indicating that there was a low level of resistance which occurred independently of T cell function. This may have been due to the action of activated macrophages. However the loss of adult worms arising from the immunising infections was highly dependent upon T cell function. Surprisingly however even the mice which had been injected with lymphoid tissue before the administration of the immunising infections failed to develop full resistance, had low granulomata forming ability and failed to expel their adult worms. The implications of this will be discussed later. The infected nu/+ mice had characteristic extremely high serum $IgG_1$ concentrations which was again dependent upon the mice having a full complement of T cells. The $IgG_{2a}$ levels of the infected nu/+ mice were very low which was probably due to an increased overall rate of catabolism of IgG (Mitchell, personal communication, unreported results). In the mice in which the IgG levels

are lower, i.e. the cell injected mice the catabolic rate of IgG would be less and hence the $IgG_{2a}$ levels a little higher than in infected nu/+ mice. This is an indication that the infection seems to specifically switch on production of only one immunoglobulin isotype, and not affect the others. This will be discussed in more detail later.

# CHAPTER 7

## The adherence of peritoneal exudate cells to third stage larvae: the role of complement and immunoglobulin

1. Introduction
2. Factors involved in the adherence of peritoneal exudate cells from normal mice to third stage larvae
3. The role of antibody in promoting adherence in sera from animals immunised with killed $L_3$ and *N. dubius* adult worm extract
4. The adherence of peritoneal exudate cells from mice resistant to reinfection to third stage larvae
5. Immunoglobulins on the surface of peritoneal exudate cells from previously infected mice resistant to reinfection
6. Conclusions.

1. Introduction

The investigations in Chapter 4 showed clearly that in resistant LACA mice previously infected with N. dubius, the worms were killed while still in the larval stage following their penetration of the intestinal mucosa. It was considered that antigens on the surface of the cuticle of the larvae may be important targets in the expression of host resistance.

In initial experiments it was found that serum from normal mice would promote the adherence of normal peritoneal exudate cells to $L_3$. It was possible that this adherence may have been due to natural antibody or to complement fixation by the cuticle of the larvae.

2. Factors involved in the adherence of peritoneal exudate cells from normal mice to third stage larvae.

To investigate the nature of factors involved in adherence,assays (according to the method given in Materials and Methods section 8) were carried out on normal sera, sera which had been heated at $56^o$ for 30 minutes to destroy complement activity, sera in the presence of E.D.T.A. which chelates $Ca^{++}$ and $Mg^{++}$ and prevents complement activation and finally, sera in the presence of E.G.T.A. which preferentially chelates $Ca^{++}$ and prevents activation of complement by the classical pathway but allows activation by the alternate pathway. Normal guinea pig serum, which is known to have high levels of haemolytic complement and serum from mice immune to reinfection were assayed also for their ability to promote cell adherence to the infective larvae under these various

conditions. Adherence which was promoted by heated sera or occurred in the presence of E.D.T.A. would be due to antibody whereas adherent activity which was abolished by either heating the serum or in the presence of E.D.T.A. would be due to complement fixation. The results of these titrations are shown in Table 7.1.

The adherence titre of the normal mouse serum was 1/128. This activity was abolished by titration of the serum in the presence of E.D.T.A. and by heat treatment suggesting that complement activation was responsible for this activity. Titration of normal mouse serum in the presence of E.G.T.A. reduced the titre but did not abolish adherence suggesting that the adherent activity was the result of the activation of complement by the alternate pathway.

The titre of immune mouse serum was 1/64. In contrast to the normal mouse serum much of this activity was independent of $Ca^{++}$ and $Mg^{++}$ but was reduced by heat treatment although not abolished. The reduction in titre in the presence of E.D.T.A. indicated that complement activation may contribute to the adherence promoted by immune mouse serum. The treatment of serum at $56^{o}$ for 30 minutes may denature some antibody as well as preventing complement activation and may well account for the decrease in titre to 1/8. Indeed, data obtained by Ey (personal communication) suggested that $IgG_1$ as well as IgE mediated passive cutaneous anaphylactic reactions were markedly reduced by heating at $56^{o}$. A further possibility is that complement and antibody may act synergistically in promoting cell adherence. A mechanism such as this which

Table 7.1

The ability of various sera to promote the adherence of normal peritoneal exudate cells to $L_3$ after certain treatments

| Treatment of sera+ | Source of serum | | |
|---|---|---|---|
| | Normal mouse | Immune mouse* | Guinea Pig |
| | Adherence titre‡ | | |
| None | 1/128 | 1/64 | 1/256 |
| Diluted in the presence of E.D.T.A. 0.01M. | 1/2 | 1/32 | 1/2 |
| Diluted in the presence of E.G.T.A. 0.01M. | 1/32 | 1/32 | 1/64 |
| Heated 56°/30 minutes | <1/2 | 1/8 | <1/2 |

\+ Diluent, Veronal buffered saline containing $Ca^{++}$ and $Mg^{++}$ as described in Materials and Methods.

* Serum from mice bled on day 35-45 following 3 doses of 200 $L_3$ orally on days 0, 14, 28.

‡ Maximum dilution at which >50% of the $L_3$ had >10 adherent cells.

results in the adherence of cells to the cuticle of schistosomulae has been described by Tavares et al., (1978).

Although the adherent activity of immune mouse serum was reduced by heat treatment and by titration in E.D.T.A. there was a portion of the activity remaining which was independent of complement fixation. It seemed likely that this was due to the binding of specific antibody in the immune mouse serum to antigens on the cuticle of the $L_3$ and the resultant binding of macrophages via their Fc receptor. The low (1/8 - 1/32) titre suggested that the amount of antibody in the serum was low. The number of antigenic sites on the cuticle may also be low since Ey (personal communication) was unable to elute immunoglobulin from the worms following incubation in sera from immune mice nor detect immunoglobulin on the larval surface using $^{125}$I-labelled anti-light chain antiserum.

The fact that the adherent activity promoted by normal guinea pig serum was abolished by titration of the serum in the presence of E.D.T.A. or by heat treatment but only reduced in the presence of E.G.T.A. indicated again that the larvae were able to activate complement by the alternate pathway. It was interesting to note that normal guinea pig serum had an adherence titre of 1/256, only twice that of normal mouse serum despite the fact that there was a greater than 100 fold difference in haemolytic complement levels,(Kindly titrated by Dr. Ey, Dept. Microbiology and Immunology, University of Adelaide). This indicated that the component of the complement pathway which is limiting this reaction must be present at similar levels in both normal mouse serum and normal guinea pig serum.

3. The role of antibody in promoting adherence in sera from animals immunised with killed $L_3$ and N. dubius adult worm extract.

Attempts made during the course of this study (not reported) and by Chaicumpa et al., (1977) to protect mice against N. dubius infection by immunisation with killed larvae, extracts of adult worms and secretions of adult worms were all unsuccessful. The above results showed that serum from mice immunised with living larvae could promote the adherence of normal peritoneal exudate cells to $L_3$ in the presence of E.D.T.A. In view of the inability of these non living vaccines to give protection it seemed possible that sera from mice immunised with these vaccines lacked the specific antibodies required to promote adherence.

To raise an anti-killed $L_3$ antiserum mice were injected subcutaneously with 2 doses of 1,000 freeze dried $L_3$ 10 days apart and bled 10 days after the final injection. The other antisera tested was produced by 5 fortnightly intravenous or intraperitoneal injections of 0.2 ml of a soluble phosphate buffred saline extract of a homogenate of adult worms (4.9 mg/ml). The mice were bled 2 weeks after the final injection.

When tested for adherence both the anti-killed $L_3$ serum and the anti-adult extract serum had low levels of antibody (Table 7.2). These levels tended to be lower than those observed in serum from mice immunised orally with 3 doses of 200 living $L_3$, (resistant mice).

These results show clearly that these sera contain antibodies directed against cuticular antigens.

Table 7.2

Cell adherence titres of antiparasite sera

| Sera from mice immunised with | adherence titre* |
|---|---|
| Killed $L_3$ + | 1/8 |
| adult worm extract ‡ | 1/4 |

* Assayed as described (Chapter 2 section 8 ) in 0.01M. E.D.T.A.

\+ Bled 10 days after the final of 2 doses of 1,000 freeze dried $L_3$ given subcutaneously 10 days apart.

‡ Bled 2 weeks following the final dose of 5×0.2ml fortnightly injections of a soluble P.B.S. extract of a homogenate of adult worms (4.9 mg/ml).

4. The adherence of peritoneal exudate cells from previously infected mice resistant to reinfection.

Since serum from mice, resistant to reinfection contained antibodies which promoted the adherence of normal peritoneal exudate cells to $L_3$ it was important to know whether peritoneal exudate cells from these mice could adhere to $L_3$ in the absence of serum.

Peritoneal exudate cells were collected from mice immune to reinfection as described (Chapter 2 section 6). These cells were washed by centrifuging at 500 g for 5 minutes and resuspended at a concentration of $2\times10^6$/ml. When these cells were mixed with $L_3$ there was strong adherence to the cuticle, even in the absence of serum. This indicated that these cells may have antibody on the surface which will bind to the cuticle of the larvae.

In order to investigate the nature of this adherence further, the peritoneal exudate cells from immune mice were incubated at a concentration of $5\times10^6$/ml in 199 culture medium supplemented with 10% heat inactivated foetal calf serum, (199+F.C.S.) containing 0.05% (w/v) trypsin (2×crystalised, Sigma) for 120 minutes at $37^{\circ}$. The cells were washed twice in 199+F.C.S. and resuspended to a final concentration of $2\times10^6$/ml.

These trypsin treated cells failed to adhere to $L_3$ in the absence of serum, which suggested that treatment with trypsin had removed the factors from the cell surface which had mediated adherence.

If immune mouse serum and normal mouse serum were titrated for all adherence promoting antibody in the presence

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of E.D.T.A. using the trypsin treated peritoneal exudate cells, only immune mouse serum contained the factors which would promote adherence. The immune mouse serum had the same adherence titre (1/16) irrespective as to whether the titration was done using trypsinised immune peritoneal exudate cells or normal peritoneal exudate cells. These results suggested that there were antibodies directed against antigens on the cuticle of $L_3$ in the serum of mice immune to reinfection, as well as on the surface of peritoneal exudate cells from these mice. The data also show that normal macrophages may bind to the antibody-larval complex as efficiently as the trypsinised peritoneal exudate cells from resistant mice since a similar adherence titre was obtained when the same serum was titrated with the 2 different cell populations.

5. Immunoglobulins on the surface of peritoneal exudate cells from mice resistant to reinfection.

The work described above showed that peritoneal exudate cells from resistant mice adhered to $L_3$ in the absence of serum. This suggested that these cells may have cytophilic antobody on their surface.

The quantity and class of immunoglobulin on the surface of peritoneal exudate cells was determined by R.I.A. (Chapter 2 section 14). The peritoneal exudate cells were collected from normal mice or mice which had received 3 doses of 200 $L_3$ orally 14 days apart (resistant mice). Cells were collected 7-10 days after the final administration of $L_3$. Ten fold less resistant mice than normal mice were used to collect peritoneal exudate cells since the resistant mice had greatly increased

total leukocyte numbers (Chapter 5). Peritoneal exudate cells from resistant mice were assayed for surface $IgG_1$ since this isotype is present in the serum of resistant mice in large quantities. The other major serum immunoglobulin isotypes in mice, $IgG_{2a}$ and IgM were also assayed. For comparison, normal mouse peritoneal exudate cells were assayed for $IgG_1$ and $IgG_{2a}$. Because of the number of mice involved and the fact that no IgM was detected on peritoneal exudate cells from immune mice an IgM determination was not carried out on normal peritoneal exudate cells. Finally, the amount of $IgG_1$ on non-adherent peritoneal exudate cells from immune mice was determined in an attempt to define the cell type bearing $IgG_1$ in the immune peritoneal exudate cell population.

The results are shown in Table 7.3. The average number of molecules per cell was determined in the following manner. The R.I.A. for cell surface immunoglobulin showed that there were 9.99ng of $IgG_1$ on $2.25 \times 10^5$ cells. Taking the molecular weight of $IgG_1$ to be 150,000 the average number of $IgG_1$ molecules per cell is given by

$$\frac{\text{Avogadro's number} \times \text{weight of } IgG_1 (\text{ng})}{\text{molecular weight of } IgG_1 \times 10^9 \times \text{number of cells}}$$

i.e.
$$\frac{6.023 \times 10^{23} \times 9.99}{150{,}000 \times 10^9 \times 2.25 \times 10^5}$$

$$= 1.78 \times 10^5$$

The peritoneal exudate cells from resistant mice had predominantly $IgG_1$ on the surface with a mean of $44 - 178 \times 10^3$

Table 7.3

Mean number of immunoglobulin molecules per peritoneal exudate cell from resistant and normal mice.

| Cell preparation | Average no of immunoglobulin molecules cell × $10^3$ | | |
|---|---|---|---|
| | $IgG_1$ | $IgG_{2a}$ | IgM |
| Normal Peritoneal Cells+ | not detected | 11.5 | not done |
| Immune Peritoneal Cells‡ | 44 - 178 | ≤2.4 | not detected |
| Non Adherent Peritoneal Cells* | 7.5 | not done | not done |

+ Cells collected from 50 mice

‡ Cells collected from 5 mice for each determination

* Cells collected from 20 mice.

molecules/cell. A low level of $IgG_{2a}$ but no IgM was detected on these cells. In contrast the normal peritoneal exudate cells had an average of $11.5 \times 10^3$ molecules of $IgG_{2a}$ per cell but no detectable $IgG_1$. The non-adherent peritoneal exudate cells from resistant mice had an average of $7.5 \times 10^3$ molecules of $IgG_1$ per cell suggesting perhaps that most of the immunoglobulin was on the surface of the plastic adherent cells. These results indicated that the adherence of peritoneal exudate cells from resistant mice to $L_3$ was probably mediated by specific immunoglobulin on the surface of these cells and this immunoglobulin is likely to be of the $IgG_1$ isotype.

## 6. Conclusions

The above work showed that the adherence of peritoneal exudate cells to $L_3$ of N. dubius may be promoted by both complement and antibody directed against antigens on the surface of the larvae. The results strongly indicated that the cuticle can fix complement by the alternate pathway of activation and peritoneal exudate cells may bind, probably by their C3 receptor. Adherence of cells was also promoted by antibody from the serum of resistant mice. This antibody was present in the sera of animals immunised with killed $L_3$ or an extract of adult worms. Peritoneal exudate cells from resistant mice could adhere to $L_3$ in the absence of serum and this adherence was trypsin sensitive. The ability of serum from resistant mice to restore this adherence and the presence of large amounts of $IgG_1$ on the surface of peritoneal exudate cells from resistant animals strongly suggested a role for antibody in promoting adherence.

# CHAPTER 8

## The effect of cells, antibodies and complement on the infectivity of $L_3$ following incubation *in vitro*

1. Introduction
2. The effect *in vitro* of peritoneal exudate cells from resistant mice on the infectivity of $L_3$ in the presence of serum from normal or immune mice
3. The effect of adherent peritoneal exudate cells from immune mice on the infectivity of $L_3$
4. The effect of non-adherent peritoneal exudate cells from resistant mice on the infectivity of $L_3$
5. The effect of normal peritoneal exudate cells in the presence of either immune mouse serum or normal goat serum on the infectivity of $L_3$ following incubation *in vitro*
6. The role of antibody and complement in promoting cell adherence and the subsequent effect on the infectivity of $L_3$
7. Conclusions

1. Introduction

The work described in previous chapters showed that peritoneal exudate cells from resistant mice would adhere strongly to the $L_3$ of *N. dubius* and implicated activated macrophages and possibly eosinophils as being cells involved in killing $L_3$ *in vivo*. Previous work by Chaicumpa and Jenkin, (1977) showed that the infectivity of $L_3$ was not affected by incubation with serum from immune mice and peritoneal cells from normal mice. These findings were confirmed in the present study.

However the infectivity was significantly reduced if peritoneal exudate cells from resistant mice were used.

To investigate further the role of these cells in resistance to this infection it was necessary to study *in vitro* the effect on larval infectivity of the interaction between $L_3$ and peritoneal exudate cells from resistant mice, in particular to see if non-adherent cells were able to damage the larvae.

The assay used is described in detail in Chapter 2, section 9. Briefly it involved mixing $L_3$, serum and cells in a tube, incubating for 20 hours then feeding samples of the contents to mice to determine the infectivity of the larvae.

2. The effect *in vitro* of peritoneal exudate cells from resistant mice on the infectivity of $L_3$ in the presence of serum from normal or immune mice.

Initial experiments were carried out to determine the time of incubation required to produce an effect upon the infectivity of the larvae.

Tubes were prepared containing 1,000 $L_3$, heat inactivated serum from either mice immune to reinfection or normal mice and peritoneal exudate cells from mice resistant to reinfection. The control tubes contained no cells and normal mouse serum. The infectivity of $L_3$ following incubation was determined as described in Chapter 2 section 9. In some experiments control tubes containing immune mouse serum or 199 medium plus 10% heat inactivated foetal calf serum (199+F.C.S.) were used. During the incubation of $L_3$ at $37^{o}$ a slow reduction in their infectivity occurred. There was no difference in the infectivity of the larvae when incubated in 199+F.C.S. alone or in the presence of immune mouse serum or normal mouse serum, normal or heat inactivated.

The results in Table 8.1 show that no significant loss in infectivity had occurred by 6 hours but had after 20 hours incubation. This experiment was repeated several times with similar results. In future experiments 20 hours was used as the standard incubation time. The data also showed that peritoneal exudate cells from immune mice were able to damage the larvae irrespective of the source of serum. This indicated that cytophilic antibody,previously described on these cells may play an important role in this phenomenon.

3. The effect of adherent peritoneal axudate cells from immune mice on the infectivity of $L_3$

Since peritoneal exudate cells from mice resistant to reinfection could reduce the infectivity of $L_3$ *in vitro* it was important to know which cell type was responsible.

Table 8.1

Reduction in the infectivity of _N. dubius_ $L_3$
by peritoneal exudate cells from animals
resistant to reinfection

| Larvae incubated in the presence of | Time of incubation (hours) | | |
|---|---|---|---|
| | 0 | 6 | 20 |
| | number of cysts+ | | |
| normal mouse serum | 83 ± 3 | 56 ± 6 | 69 ± 5 |
| normal mouse serum $10^7$ peritoneal cells‡ | N.D.* | 45 ± 3 | 40 ± 3# |
| immune mouse serum $10^7$ peritoneal cells | N.D. | 57 ± 3 | 35 ± 3# |

+ Arithmetic mean of 6 mice ± standard error

* N.D. = not done

‡ Peritoneal exudate cells from immune mice

# Probability, 'U' test of Mann and Whitney

6 hours no significant difference

20 hours normal mouse serum vs normal mouse serum + cells

P=0.004

normal mouse serum vs immune mouse serum + cells

P=0.004

Monolayers of peritoneal exudate cells from resistant mice were prepared as described in Materials and Methods, section 6 . Microscopic examination showed that these cells were predominantly macrophages. To these tubes, 1.0 ml of 199 medium containing 2,000 exsheathed $L_3$ and either, 0.5 ml of heat inactivated serum from normal or resistant mice was added. After a further 20 hours incubation at $37^{o}$ the monolayers were vigorously shaken to free larvae from the cells and an 0.2 ml sample of each tube fed to a mouse. The control tubes contained the same number of $L_3$ and normal mouse serum but no cells. The results are given in Table 8.2.

The data shows clearly that the reduction in the infectivity of $L_3$ may be caused by glass adherent peritoneal exudate cells from resistant mice and was independent of the source of serum.

4. The effect of non-adherent peritoneal exudate cells from resistant mice on the infectivity of $L_3$

The previous experiment showed that adherent peritoneal exudate cells from resistant mice could reduce the infectivity of $L_3$ _in vitro_. In order to determine if other cells from the peritoneal cavity of resistant mice could kill $L_3$, non-adherent cells obtained from the peritoneal cavities of resistant mice were cultured with $L_3$.

Peritoneal exudate cells were collected from resistant mice as described earlier. They were allowed to settle onto plastic petri dishes for 30 minutes at $37^{o}$. After incubation the dishes were agitated gently and the non-adherent cells and the 199 medium removed. A further 2 ml of ice cold

Table 8.2

The reduction in the infectivity of *N. dubius* $L_3$
by glass adherent peritoneal exudate cells
from mice resistant to reinfection

| Larvae incubated in the presence of | Number of tubes | Number of cysts* | Probability‡ |
|---|---|---|---|
| Normal mouse serum | 6 | 131 ± 5 | - |
| Normal mouse serum + adherent peritoneal exudate# cells | 6 | 92 ± 6 | P<0.004 |
| Immune mouse serum + adherent peritoneal exudate cells | 3 | 15 ± 2 | P<0.004 |

* Arithmetic mean, ± standard error

‡ Probability determined by 'U' test of Mann and Whitney

\# Peritoneal cells from mice resistant to reinfection.

199+F.C.S. was added to the dishes which were agitated once again following which this 199+F.C.S. containing cells was removed. The media containing non-adherent cells (collected from the dishes) were pooled and the cell preparations washed by centrifuging. Finally the cells were resuspended in 199+F.C.S. at a concentration of $1.3 \times 10^7$ cells/ml. The cells were mostly eosinophils, neutrophils and lymphocytes as determined by differential cell counts on smears stained with Wrights Blood Stain. The cell preparation had very few macrophages (<5%).

Three tubes were prepared in the following manner. Tube A, the control tube contained 0.5 ml of medium, 2,000 $L_3$ in 0.5 ml of medium and 0.5 ml of heat inactivated serum from resistant mice. Tubes B and C contained either $6.5 \times 10^6$ cells from the pool of non-adherent peritoneal exudate cells in 0.5 ml of 199+F.C.S. (B) or $8.5 \times 10^6$ peritoneal exudate cells from resistant mice in the same volume (C). To tubes B and C were added 2,000 $L_3$ in 0.5 ml of 199+F.C.S. plus 0.5 ml of heat inactivated serum from resistant mice. The tubes were incubated at $37^{\circ}$ for 20 hours and the infectivity determined as described (Chapter 2 section 9).

The results in Table 8.3 show that peritoneal exudate cells which did not adhere to plastic also had the ability to reduce the infectivity of $L_3$. These cells are as effective at reducing the viability as were the mixture of adherent and non adherent cells (Tube C).

Table 8.3

The reduction in the infectivity of N. dubius $L_3$ by non-adherent peritoneal exudate cells from mice resistant to reinfection

| Larvae incubated in the presence of | Number of cysts* | Probability‡ |
|---|---|---|
| (A) Immune mouse serum | 142 ± 8 | - |
| (B) Immune mouse serum $6.5 \times 10^6$ non adherent peritoneal exudate cells+ | 102 ± 8 | <0.004 |
| (C) Immune mouse serum $8.5 \times 10^6$ peritoneal exudate cells+ | 104 ± 8 | <0.004 |

\+ Peritoneal exudate cells from resistant mice

* Arithmetic mean of 6 mice ± standard error

‡ Determined by the 'U' test of Mann and Whitney.

5. The effect of normal peritoneal exudate cells in the presence of either immune mouse serum or normal goat serum on the infectivity of $L_3$ following incubation *in vitro*.

It has been demonstrated by several workers that incubating macrophages *in vitro* in heterologous sera may result in their activation,(Cohn and Benson, 1966; Hibbs et al., 1977). If activated macrophages were involved in the loss of infectivity of $L_3$ following incubation *in vitro* with peritoneal exudate cells from resistant mice then incubation of normal macrophages in the presence of a heterologous sera may activate them to a state where they may affect the infectivity of $L_3$.

To investigate this possibility experiments were carried out *in vitro* where mixtures of normal goat serum, immune mouse serum, normal peritoneal exudate cells and $L_3$ were incubated together. If the above concept of activation was correct then the peritoneal exudate cells, activated by the normal goat serum should affect $L_3$ in the presence of serum from immune mice.

Three experiments were carried out with similar results being obtained in each. The details of one such experiment is outlined below. After washing, the peritoneal exudate cells from normal mice were resuspended in either normal mouse serum or serum from resistant mice at a concentration of $2.6\times10^7$/ml. All sera used were heat inactivated. Four tubes were prepared. Tube 1, the control tube contained 0.5 ml of normal mouse serum and 0.5 ml of immune mouse serum. Tubes 2 and 3 contained $1.3\times10^7$ peritoneal exudate cells in 0.5 ml

of immune mouse serum. In addition, tube 2 contained 0.5 ml of normal mouse serum and tube 3, 0.5 ml of normal goat serum. Finally, tube 4 contained $1.3 \times 10^7$ peritoneal exudate cells in 0.5 ml of normal mouse serum and 0.5 ml of normal goat serum. All tubes received 1,500 $L_3$ in 0.5 ml of 199+F.C.S. and following incubation for 20 hours at $37^{\circ}$ the infectivity of the larvae was determined as previously described.

The results in Table 8.4 show that the only tube in which the viability of the larvae was significantly reduced was the one which contained normal goat serum and immune mouse serum (Tube no. 3).

This strongly suggested that incubation of the peritoneal exudate cells in the presence of a heterologous sera resulted in their activation and the subsequent reduction in the infectivity of $L_3$ provided specific antibody was present.

6. The role of antibody and complement in promoting cell adherence and the subsequent effect on the infectivity of $L_3$

The results obtained in Chapter 7 showed that both antibody and complement could promote the adherence of peritoneal exudate cells from normal mice to $L_3$. In order to ascertain the importance of this adherence in causing the subsequent reduction in the infectivity of $L_3$, trypsin treated peritoneal exudate cells from resistant mice were incubated with $L_3$ in the presence of untreated and heat inactivated sera from normal and resistant mice.

Table 8.4

The effect of normal peritoneal exudate cells in the presence of normal goat serum, normal mouse serum and immune mouse serum on larval infectivity following incubation *in vitro*

| Larvae incubated in the presence of | | Cysts[+] ± S.E. | P[‡] |
|---|---|---|---|
| Serum | Cells* | | |
| 1. immune mouse<br>normal mouse | none | 106 ± 4 | Control |
| 2. immune mouse<br>normal mouse | $1.3\times10^7$ | 104 ± 7 | N.S. |
| 3. immune mouse<br>normal goat | $1.3\times10^7$ | 82 ± 3 | 0.001 |
| 4. normal mouse<br>normal goat | $1.3\times10^7$ | 105 ± 5 | N.S. |

\+ Arithmetic mean ± standard error of 6 mice

‡ Probabilities determined by 'U' test of Mann and Whitney

N.S. = not significant $P>0.05$

* Peritoneal exudate cells from normal mice.

Tubes were prepared as outlined below. Tube A contained $8.5\times10^6$ peritoneal exudate cells from immune mice in 0.5 ml of 199+F.C.S., 1,000 $L_3$ in 0.5 ml of 199+F.C.S. and 0.5 ml of heat inactivated normal mouse serum. Tubes B,C,D and E each contained $8.5\times10^6$ peritoneal exudate cells from mice, resistant to reinfection which had been incubated in 0.05% trypsin at $37^o$ for 90 minutes, washed and resuspended in 199+F.C.S. as well as 1,000 $L_3$ in 0.5 ml of 199+F.C.S. To tube B, 0.5 ml of heat inactivated normal mouse serum was added; tube C, 0.5 ml of untreated normal mouse serum; to tube D, 0.5 ml of untreated immune mouse serum and to tube E, 0.5 ml of heat inactivated immune mouse serum. Tube F, a control tube contained 1,000 $L_3$ in 0.5 ml of 199+F.C.S., 0.5 ml of 199+F.C.S. and 0.5 ml of heat inactivated normal mouse serum. All tubes were incubated for 20 hours at $37^o$ and the reduction in the infectivity of the larvae determined.

The results in Table 8.5 show that trypsin treatment of peritoneal exudate cells from resistant mice abolished their ability to cause a reduction in infectivity. However this could be restored by adding untreated serum from normal mice or either heat treated or untreated serum from mice resistant to reinfection.

This indicates that complement as well as antibody may play a role in promoting cell adherence leading to a reduction in the infectivity of the larvae.

## 6. Conclusions

Data presented in this chapter show that both adherent and non-adherent peritoneal exudate cells from mice resistant

Table 8.5

The role *in vitro*, of antibody and complement in the presence of peritoneal exudate cells from immune mice on the infectivity of $L_3$

| Tube | Larvae incubated in the presence of | | Cysts S.E.* |
|---|---|---|---|
| | Cells | Serum | |
| A | P.E.C.[+] immune mice | Heated[‡] normal mouse | 66 ± 4** |
| B | P.E.C. immune mice trypsin treated | Heated normal mouse | 80 ± 6 |
| C | P.E.C. immune mice trypsin treated | Normal mouse | 53 ± 5 |
| D | P.E.C. immune mice trypsin treated | Immune mouse | 56 ± 5 |
| E | P.E.C. immune mice trypsin treated | Heated immune mouse | 59 ± 3 |
| F | none | Heated normal mouse | 82 ± 3 |

* Mean of 6 mice ± standard error

\+ P.E.C. = Peritoneal exudate cells

‡ Sera heated 56$^{o}$ for 30 minutes

** Statistical analysis, 'U' test of Mann and Whitney

A vs F, P=0.008 B vs D, P=0.013

B vs F, not significant B vs E, P=0.004

B vs C, P=0.004

to reinfection posses the ability to reduce the infectivity of $L_3$ in vitro in the absence of serum from resistant mice. This implies that the antibody on the surface of these cells which was shown previously to be predominantly $IgG_1$, is of importance in the expression of resistance to this infection. The adherent cells, which were predominantly macrophages may be activated since it has been shown that normal peritoneal exudate cells which are almost all macrophages have no effect upon the larvae even in the presence of serum from immune mice. This is supported by the ability of cells, incubated in heterologous serum to reduce the infectivity of $L_3$ following in vitro incubation in the presence of serum from mice resistant to reinfection.

It is clear also that cells present in the population of non-adherent peritoneal exudate cells from immune mice may play some part in causing the reduction in infectivity of the $L_3$.

The results also indicate that complement activation may cause adherence of cells to larvae and providing these are from mice resistant to reinfection there was a subsequent loss in the infectivity of the $L_3$.

# CHAPTER 9

## Discussion

The present study has examined in detail the host/parasite relationship between the mouse and the intestinal nematode N. dubius. Initial investigations indicated that some strains of mice developed a high level of resistance following prior infection while others did not. It was also evident that within some strains of mice female mice developed better resistance than male mice.

Resistance seemed to be expressed in a number of different ways, namely

(1) the ability of mice to kill larvae in the intestinal mucosa before they encyst;

(2) the accumulation of cells around larvae in the intestinal wall; and

(3) the expulsion of adult worms from the intestinal lumen.

These 3 mechanisms may not necessarily all operate in any one strain of animal, for example, LACA mice had the ability to develop a high degree of resistance to a challenge dose of larvae and mount an inflammatory response as evidenced by the large numbers of granulomata in the intestinal wall. However these mice were unable to rid themselves of adult worms. In contrast to this the C3H/HeJ mice resisted a challenge dose of $L_3$ following immunisation, mounted a good inflammatory response and

rid themselves of adult worms. On the other hand CBA/H mice, neither developed good resistance to a challenge infection, did not mount a good inflammatory response, nor expelled their adult worms. There was found to be a good inverse relationship between the numbers of cysts and granulomata in the inbred immunised mice, and when this was expressed as a ratio it proved to be a very sensitive index of resistance and correlated well with the numbers of adult worms found in the immunised inbred mice, for example, C3H/HeJ female mice had a cyst to granulomata (C:G) ratio of 0.036 and a mean of 4 adult worms whereas CBA/H male mice had a C:G ratio of 1.51 and a mean of 312 adult worms.

The rate of loss of adult worms from inbred mice was in contrast to that observed by other workers. The loss observed here was slow but steady following one infection with the rate probably faster if several doses of $L_3$ were given. There was obviously no sudden expulsion as seen in N. brasiliensis infections or no rapid 'self cure' following the challenge of immune mice with $L_3$, as observed by Cypess and Van Zandt, (1973) and Cypess and Zidian, (1975). The loss resembled more that described by Cypess et al., (1977); Liu, (1966) and Dobson and Owen, (1977) although there were large differences in the rate of loss observed by these investigators. The rate of loss of worms may vary considerably between experiments as indicated by the results reported here. The rate of loss of adult worms from the intestines of mice was possibly slightly

faster if they had arisen as a result of a larval infection rather than if they had been artificially planted there by oral administration. It was also evident that although LACA mice developed good resistance to reinfection and had high numbers of granulomata they could not expel their adult worms. A similar situation has been described by Dobson and Owen, (1977) who showed that the rate of loss of adult worms was slower in Quackenbush mice in which the parasite had been maintained. In this department N. dubius has been maintained in LACA mice for at least 10 years.

The failure of nude mice to expel adult worms which accumulated from the immunising infections suggested that the ability of mice to rid themselves of adult worms was highly T cell dependent. However nude mice which had received lymphoid tissue from intact mice were unable to expell adult worms. These cell injected nude mice did not respond as intact animals as measured by a further parameter since, after multiple infections they did not have serum $IgG_1$ concentrations similar to those seen in infected intact mice. It is difficult to explain this situation but it is possible that the nude mice may have received an insufficient number of T cells or failed to receive a particular subset of T cells required for the development of full resistance.

A similar situation to this has been described by Wakelin and Selby, (1974) who found that T cell depleted mice failed to expel completely T. muris following the

injection of lymphocytes from intact animals.

Nude mice appear unable to expel a number of other intestinal helminths. Studies have shown that N. brasiliensis, (Jacobson and Reed, 1974, 1976; Mitchell et al., 1976 ), T. spiralis, (Ljungstrom and Ruitenberg, 1976; Ruitenberg et al., 1977), T. muris, (Wakelin and Selby, 1974) and H. nana, (Isaak, Jacobson and Reed, 1977), all persist in nude or T cell depleted mice.

A brief study of the inheritence of the ability to develop resistance to N. dubius in the $F_1$ progeny of crosses between strains of mice which developed high resistance, (C3H/HeJ) and mice which developed poor resistance, (CBA/H) was carried out. It was demonstrated that the progeny which received the Y chromosome from the strain of mice which developed poor resistance, (CBA/H) themselves developed a low level of resistance. In contrast the male $F_1$ mice which received the Y chromosome from the resistant C3H/HeJ strain developed good resistance. The female $F_1$ progeny of either cross all had similar but higher levels of resistance than the males. Thus it is clear that the failure of some strains of mice to develop good resistance was linked to the Y chromosome. This is illustrated in Table 9.1.

There are a number of possible explanations for this observation. It has been suggested by Damian, (1978) that there is some sharing of antigens between N. dubius and some strains of mice and this may explain the failure of these strains of mice to develop good resistance. He

Table 9.1

The genotype of the progeny of a cross between C3H/HeJ and CBA/H mice

| | Parental strains | | $F_1$ Progeny | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| | C3H/HeJ | CBA/H | | |
| Genotype | XY | XX | XY | XX |
| Observed resistance | good | good+ | good | good |
| C:G ratio‡ | 0.21 | 0.84 | 0.15 | 0.16 |
| | CBA/H | C3H/HeJ | | |
| Genotype | XY | XX | XY | XX |
| Observed resistance | poor | good | poor | good |
| C:G ratio | 3.1 | 0.14 | 2.5 | 0.27 |

\+ As well as sex linked differences there are also strain differences since female mice of different strains develop different levels of resistance.

‡ From Table 3.7. Note that there is no significant difference between the C:G ratios of the 2 female parental groups.

demonstrated that C57Bl mice develop strong resistance to reinfection whereas the LP strain develops a weaker immunity. Mice of the C57Bl strain, which were made tolerant to LP histocompatability antigens showed a significantly reduced ability to reject a parasite challenge infection. He also demonstrated that C57Bl mice, immunised against *N. dubius*, rejected LP allografts at an accelerated rate although this effect was not noted with C3H allografts. If this were so and the genes controlling the production of these antigens were linked to the Y chromosome then this could explain the results obtained here. The gene products would be expressed in the male CBA/H mice and hence prevent these mice from developing an immune response against the shared antigens. The C3H/HeJ mice, not expressing the same gene products would be able to mount an immune response against these parasite antigens and hence may be better protected. Other explanations such as the presence of genes linked to the Y chromosome controlling the production of blocking antibody or the production of a factor which may partially suppress the immune response to *N. dubius* are purely speculative and as indicated above, there is only evidence for the first explanation.

As outlined previously, one of the characteristic responses of animals infected with helminths is the production of antibody which may bind to mast cells and elicit immediate type hypersensitivity (I.T.H.) reactions. Many workers have suggested that the I.T.H. reaction may play a role in the expulsion of parasites from the intestine.

Panter, (1969b) proposed that this mechanism resulted in the expulsion of N. dubius larvae from the intestine of resistant mice. Jones and Rubin, (1974) counted larvae in enzyme digests of intestinal homogenates from mice following an oral larval challenge. They recovered less larvae from challenged mice which had been immunised by oral administration of $L_3$ than from challenged mice which had been immunised by the subcutaneous administration of $L_3$, when compared with the recovery from challenged naive mice. This supported the concept that in mice immunised orally the larvae of the challenge dose were expelled from the intestine, presumably by an I.T.H. reaction whereas in mice immunised subcutaneously the larvae penetrated the intestinal wall where they were killed. To obtain a better understanding of the mechanisms of immunity one must ascertain whether different routes of immunisation induce different mechanisms of immunity. The technique of counting larvae in mice following challenge as used by these authors was attempted in the present study but due to poor recovery was found to be too unreliable to be of use.

It was known that when the larvae penetrated the small intestine cellular accumulation would result in the formation of a granuloma around the worm. By determining the increase in the number of granulomata in immune mice following oral challenge by $L_3$ it was shown that, irrespective of the mode of immunisation, the $L_3$ used in the challenge dose penetrated the intestinal wall. The increase in the

number of granulomata was closely related to the number of $L_3$ in the challenge dose, indicating that almost all of the larvae had penetrated.

Further experiments using the antihelminthic drug pyrantel, which acts on the adult worms in the lumen of the intestine and not on the larvae after penetrating the intestinal wall, showed that the stage of larval development in the intestinal mucosa played an important role in the development of resistance. Chaicumpa, (1973) and Bartlett and Ball, (1972) using LACA mice or C.F.W. mice respectively, found that adult worms, transplanted to the small intestine did not induce resistance. These results indicated that adult worms were not necessarily required for the induction of resistance. This will be discussed later in more detail.

A similar situation has been described for the dwarf tapeworm of man and animals, Hymenolepis nana. Heyneman, (1962) found that protection developed primarily in response to the tissue stage of infection. The larvae penetrate the intestinal mucosa and undergo a period of growth and development emerging as an adult in the lumen 5 days later. It is also this stage which is killed by mice, immune to reinfection. Infection with cystercercoids, where no tissue penetration occurs, provided no protection against a second challenge infection.

Infection of LACA mice by N. dubius induced changes in total leukocyte number and type and in the $IgG_1$ concentration of the serum. Following one infection the

number of lymphocytes, neutrophils and monocytes in the blood increased as did the number of lymphocytes and macrophages in the peritoneal cavity. Neutrophils also appeared in the peritoneal cavity. Eosinophils did not appear in the blood or peritoneal cavity until after a second infection, despite the presence of increased numbers of eosinophils in the bone marrow of mice which had undergone one infection.

The appearance of eosinophils in the blood and peritoneal cavity only after a second infection although in agreement with the leukocyte changes described by Baker, (1962) is atypical when compared with the induction of eosinophilia following other helminth infections. For example, Basten et al., (1970) observed an eosinophilia in the blood of rats 9 days after one oral T. spiralis infection and four days after the intravenous injection of an extract of T. spiralis. Eosinophils have been observed in the circulation of mice 5 days after a single infection with S. mansoni, (Phillips et al., 1977), 12 days following infection of rats with T. taeniaeformis, (Ansari and Williams, 1975) and 10 days following a primary infection of mice with A. suum, (Nielson, Fogh and Andersen, 1974). The reason why mice required a second infection is not clear. The results presented here suggest that one infection stimulated eosinophil production in the bone marrow and a second stimulus was required to elicit a peripheral eosinophilia. Animals carrying adult worms following a single larval infection do not develop an eosinophilia,

even several months after infection (Jenkin, personal communication), which suggests that the stimulus resulting in an eosinophilia depends on the larval rather than the adult worm stage of the infection.

A single infection also induced an increase in the $IgG_1$ concentration of the serum. An extraordinarily high concentration of between 20-50 mg/ml was reached following a second infection. The other isotypes were not stimulated by N. dubius infection, and in some cases remained extremely low, probably due to the high catabolic rate of IgG in infected animals.

The $IgG_1$ levels observed here are consistant with those observed by Crandall, Crandall and Franco, (1974). Serum $IgG_1$ levels of this nature have also been observed in mice with other chronic hemlinth infections such as S. mansoni, (Sher et al., 1977), M. corti, (Mitchell et al., 1977) and T. taeniaeformis, (Mitchell, Goding and Richard, 1977). The adult N. dubius may play some role in stimulating production of $IgG_1$ but the larval stages alone are certainly able to induce production of these extremely high levels.

The studies in nude mice indicated that the $IgG_1$ production was T cell dependent. What proportion of this $IgG_1$ is directed against parasite antigens is not known. Double diffusion assays (not reported here) using $IgG_1$ isolated from the serum of immune mice and a soluble extract of adult worms showed that there was anti-parasite antibody associated with this isotype. Preciptin lines could be detected at $IgG_1$ dilutions of 1/16 of the original serum

concentration. The $IgG_1$ from immune mice also had the ability to elicit 2 hour P.C.A. reactions in mice using the soluble extract of adult worms, (Ey et al., 1978). However since immunisation of mice with this extract was unable to induce protection, the role of these antibodies directed against adult worm antigens in resistance to this infection is not clear.

In another helminth infection Sher, McIntyre and Von Lichtenberg, (1977) estimated that an extraordinarily high level of 30% (> 10 mg/ml) of the $IgG_1$ produced in response to S. mansoni infection was specific antibody directed against parasite antigens. The mechanism of stimulation of this $IgG_1$ synthesis is not clear and further studies to elucidate the factors which control the production of the immunoglobulin isotypes during helminth infections needs to be done.

The protection against a larval challenge infection in LACA mice seemed to develop in 2 stages. A single infection induced partial (approximately 50%) protection in the absence of an eosinophilia. A second infection resulted in almost complete protection. The development of this protection correlated well with the appearance of eosinophils in the blood and peritoneal cavity as well as the peak of the $IgG_1$ response.

Results not reported here and those of Panter, (1969a); Cypess, (1970); Chaicumpa, Jenkin and Rowley, (1977) showed that serum from mice, resistant to reinfection was unable to provide protection against a

larval challenge when transferred to normal mice, despite the use of large volumes of serum. However if peritoneal exudate cells and serum from resistant mice were transferred, a significant degree of protection was obtained (unreported results). These latter results together with the fact that nude mice developed a partial resistance to reinfection following exposure to $L_3$ suggested that an important component of immunity to this infection resided in the cells. One possibility seemed to be an alteration in the activity of the phagocytic cells, particularly the macrophages since nude mice are unable to produce an eosinophilia when infected with helminths, (Hsu et al., 1976; Ruitenberg et al., 1977 and Mitchell et al., 1977) and are known to have activated macrophages, (Cheers and Waller, 1975) as evidenced by increased initial resistance to intracellular bacterial parasites, (Nichol and Bonventre, 1977) and the protozoan parasite, Trypanasoma rhodesiensie, (Campbell, Esser and Phillips, 1978). It seemed possible that the nude mouse may have produced some antibody directed against larval antigens. This antibody, in combination with the activated macrophages may be sufficient to kill some of the larvae of a challenge dose. This is supported by the ability of serum from mice immune to reinfection by N. dubius to provide partial protection against a larval challenge when transferred to naive nude mice, despite providing no protection when transferred to intact mice. This may be similar to the mechanism which results in the partial protection obtained in LACA mice following one infection.

The nature of the protective antibody(ies) is at

present not known. However certain experiments indicated that those of the $IgG_1$ isotype are of importance. This particular isotype, isolated from the serum of LACA mice almost completely immune to reinfection, enhanced immunity when transferred to LACA mice, partially immune to reinfection. This suggested that the presence of antibody of this isotype may be an important factor in the expression of resistance.

The investigations in the nude mice showed that granulomata formation was highly dependent upon the animal having a full complement of T lymphocytes. This is in agreement with the observations of Bartlett and Ball, (1974) who showed that mice, depleted of T cells by thymectomy and irradiation and reconstituted with bone marrow also lacked the ability to form granulomata. However in contrast to these findings granulomata around eggs of S. mansoni in the nude mouse have been reported by Hsu et al., (1976); Bryam and Von Lichtenberg, (1977) and Phillips et al., (1977), although they were reduced in size and the cellular infiltration was not as intense as that observed in intact mice. It seemed likely that the formation of granulomata of sufficient intensity to become visible, may not be an absolute requirement for the killing of N. dubius larvae since previously infected nude mice were partially resistant to reinfection but had no granulomata.

In an attempt to define which cell types might be involved in damaging the larvae, various combinations of cells and sera were incubated in vitro together with the

$L_3$ and the effect upon larval infectivity determined. It was apparent from the results of these experiments that both adherent and non-adherent peritoneal exudate cells from resistant mice reduced the infectivity of $L_3$ *in vitro*, even in the absence of serum from either immune or normal mice. However peritoneal exudate cells from normal mice, which were almost all macrophages, had no effect upon infectivity, even in the presence of serum from immune mice. The adherent peritoneal exudate cells from immune mice were predominantly macrophages and were probably 'activated' since normal macrophages could not damage $L_3$.

Further evidence implicating activated macrophages in the expression of resistance to $L_3$ came from studies showing that the incubation *in vitro* of $L_3$ with normal peritoneal exudate cells in the presence of normal goat serum and serum from immune mice resulted in reduced larval infectivity. Several studies have demonstrated that incubation of peritoneal exudate cells in heterologous serum changes their enzyme content, (Cohn and Benson, 1965) and their tumouricidal properties, (Hibbs et al., 1977).

There is also good evidence that activated macrophages may affect other helminths *in vitro*. Schistosomulae of *S. mansoni* are damaged following incubation *in vitro* with activated macrophages (Li Hsu, 1977; Capron et al., 1977; Perez and Smithers, 1977) and the viability of protoscolices of the cestode, *Echinococcus multilocularis* is reduced after incubation *in vitro* with activated macrophages from *E. multilocularis* or B.C.G. infected mice, (Baron and

Tanner, 1977). Evidence that B.C.G. treatment of animals may partially protect mice against infection by S. mansoni, (Civil, Warren and Mahmoud, 1978) and rats against E. multilocularis infection, (Reuben, Tanner and Rau, 1978) together with the studies of Perrudet-Badoux, Binaghi and Boussac-aron, (1978) and Blum and Cioli, (1978), on T. spiralis and S. mansoni infections in Biozzi high and low responder mice all suggested that activated macrophages may play a role in resistance to some helminths in vivo.

The fact that cells from the peritoneal cavity of mice immune to reinfection which did not adhere to plastic damaged N. dubius $L_3$ in vitro showed that cells other than macrophages may be involved in killing $L_3$ in vivo. The non-adherent cell population contained eosinophils, neutrophils and lymphocytes.

It was shown that peritoneal exudate cells from immune mice adhered strongly to $L_3$ in the absence of serum. This suggested that some of these cells had cytophillic antibody on their surface. This was also indicated by the R.I.A. studies which showed that the peritoneal exudate cells had an average of between 40-180,000 molecules of $IgG_1$ on their surface. Some of this $IgG_1$ may have been on the surface of B. lymphocytes in the peritoneal cavity. However the absence of $IgG_1$ on the non-adherent peritoneal exudate cells shown by R.I.A., indirectly suggested that this immunoglobulin was predominantly on plastic-adherent cells. It is possible that during the infection, macrophage receptors may be altered since the studies of Unkless and

Eisen, (1975) and Dissanayke and Hay, (1975) have indicated that under normal conditions, $IgG_1$ does not bind to mouse macrophages. However, Dissanayke and Hay, (1975) showed clearly that $IgG_1$ can bind to macrophages if the $IgG_1$ concentration is high and not in competition with $IgG_2$.

Trypsin treatment of peritoneal exudate cells from immune mice abolished their ability to adhere to the larvae and this activity could be restored by pretreating them with serum from immune mice in the presence of E.D.T.A. to prevent complement activation. Normal macrophages would also bind to larvae, sensitised in the same way. Pre-incubation of normal peritoneal cells with the immune serum followed by washing and subsequent addition to $L_3$ did not result in adherence suggesting that normal macrophages could not bind the antibody in immune mouse serum unless it had bound antigen first.

In the absence of E.D.T.A., normal serum promoted strong adherence of normal peritoneal exudate cells to the larvae. Since this adherent activity was destroyed by heating the serum at $56^{\circ}$ for 30 minutes, by titration in the presence of E.D.T.A. but only reduced by titration of the serum in the presence of E.G.T.A. it seemed likely that the cuticle of the larvae could fix complement by the alternate pathway. This would enable normal peritoneal exudate cells to bind to the cuticle of the larvae through a complement component receptor, probably C3, on the peritoneal exudate cells, (Prowse, Ey and Jenkin, 1979).

There have been a number of reports showing that the cuticle of S. mansoni may fix complement, (Machado et al., 1975; Sher, 1976; Tavares et al., 1978; Ramalho-pinto, McLaren and Smithers, 1978) and, Capron et al., (1975), Perez and Smithers, (1977), Mackenzie et al., (1977) and Butterworth et al., (1977) have demonstrated that antibody may also promote binding of cells to the cuticle of S. mansoni schistosomulae. Some of these workers have produced evidence that these mechanisms may play a role in mediating damage to schistosomulae. Evidence that the cuticle of the nematode larvae of A. suum, may fix complement in the apparent absence of specific antibody has been found by Jeska, (1973) and Ziprin and Jeska, (1975). Mackenzie, Preston and Ogilvie, (1978) have likewise shown that the infective larvae and adults of T. spiralis and larvae of N. brasiliensis may activate complement which promotes cell adherence.

It is possible that both antibody and complement together is the most efficient means of promoting adherence resulting in a reduction in larval infectivity. A situation such as this has been observed by Mackenzie et al., (1978) in their study of the viability of newborn T. spiralis larvae following incubation with various serum and cell combinations.

Despite the correlation between the development of good resistance and the appearance of eosinophils, no clear evidence was obtained as to whether eosinophils could kill N. dubius $L_3$ in vivo or in vitro. This dilemma has arisen in other helminth infections, for example, S. mansoni in mice (Knopf, 1979) and N. brasiliensis in rats (Ogilvie, Hesketh and Rose, 1978). However there is some evidence that eosinophils may be involved in damage to S. mansoni schistosomula, (Mahmoud

et al., 1975) and *T. spiralis* larvae, (Grove et al., 1977) as determined by the loss of resistance in immunised mice which had been treated with a specific anti-eosinophil serum. However these results must be interpreted with caution since there is no indication as to how the treatment of mice with anti-eosinophil serum may affect other immune functions.

It was not possible to obtain an eosinophil preparation of sufficient purity to conclude that eosinophils alone could damage $L_3$ *in vitro*. Eosinophils have been shown to be able to damage schistosomulae of *S. mansoni* following *in vitro* incubation by Butterworth et al., (1977) and Li Hsu et al., (1977).

Histological studies of the cellular composition of granulomata 3-4 days post infection in immune mice showed that they were composed predominantly of eosinophils and macrophages (Jones and Rubin, 1974; Jones, 1974). Further studies 24-48 hours following challenge of immune mice need to be carried out to determine the types of cells in intimate contact with the larvae at the time of killing.

In summary, following a primary infection of mice the larvae penetrate the intestinal wall where they start to grow. During this period of growth they probably secrete enzymes and metabolic products which may be antigenic. These secretions possibly play a role in stimulating the production of $IgG_1$ and initiating changes in cell populations since mice immunised with larvae, killed by freeze drying to minimise antigenic damage, undergo none of these changes

but still produce antibody against cuticular antigens. This study has demonstrated the importance of the tissue phase of the infection in the induction of resistance. During the penetration of the intestinal mucosa, antigens on the cuticle would also be exposed to the hosts immune system.

When the juvenile worm leaves the cyst and re-enters the intestinal lumen more antigens may be released and the accumulation of cells around the cyst debris results. The changes in cell levels could be correlated with this event occurring 7-8 days post infection.

The adult worms may be slowly lost from the intestinal lumen depending on the strain of mouse. This loss occurs at similar rates in mice which had either undergone a larval infection or received adult worms transplanted orally. This indicates that antigenic secretions of adult worms may themselves be able to stimulate an immune response resulting in their loss.

The studies in the nude mice showed that T cell involvement is required for both $IgG_1$ production and granulomata formation.

Larvae administered to mice which are immune to reinfection also penetrate the intestinal wall. These larvae probably begin to grow. It is at this stage, 24-48 hours post infection, that the hosts immune system would be likely to recognise antigens secreted by and on the surface of the larvae.

The recognition of secreted antigens by sensitised T

cells results in the release of chemotactic factors which would cause the accumulation of cells around the larvae. Complement fixation by the cuticle may also contribute to this cellular build up. In immune mice these cells would be able to kill the larvae.

The cells bind strongly to the larvae, either via antibody or complement. The antibody is likely to be of the $IgG_1$ isotype. These cells in intimate contact with the larvae 24-48 hours post infection are probably the cells which can kill the larvae. It is important to realise that the granulomata which result from a larval challenge of immune mice commence formation around a live larvae 24-48 hours post infection whereas in mice following a single infection the granulomata form around cyst debris 7-9 days post infection. It is interesting that the nude mouse may kill some of the $L_3$ despite the fact that there is no granulomata formation. There is probably a build up of cells capable of killing the larvae but not in sufficient number to form a visible granulomata.

Results obtained suggest that activated macrophages are involved in killing the larval challenge. Other cells are almost certainly involved. These may be dependent upon T cell function since full protection is T cell dependent. These cells may be eosinophils but no definitive evidence for this was obtained.

In some strains of mice the adult worms arising as a result of several infections will persist whereas they may be expelled from others. The presence of shared antigens

between the host and the worm may result in the persistence of adult worms. It may also be possible that the adult worm has adapted to resist the hosts immune response where the parasite has been maintained in the same strain of mouse for a long period of time.

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