THE ROLE OF POLYMORPHONUCLEAR CELLS IN IMMUNITY TO

NEMATOSPIROIDES DUBIUS INFECTIONS IN MICE

by

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A thesis submitted for the degree of

Doctor of Philosophy

in

The Department of Microbiology and Immunology

The University of Adelaide,

Adelaide,

South Australia

April 1984

ABSTRACT

In the present investigation, eosinophils and neutrophils purified by density gradient centrifugation from the blood of Nematospiroides dubius infected mice resistant to reinfection, were examined for their ability to adhere to the different parasitic larval stages of N. dubius. In addition, the ability of these cells to damage larvae, both in-vitro and in-vivo in the presence of immune serum was also studied.

Granulocytes were initially tested for adherence to larvae which had been sensitised with immune mouse serum or normal mouse serum in the presence of Ca^{++} and Mg^{++} ions, EDTA or EGTA. Differences were observed in the degree of cell adherence to the different stages of the parasite. However the adherence of the two cell types to any given stages of the parasite were similar. Adherence to the sheathed infective third stage larvae, 96 hr post infective larvae and to adult worms depended to a large degree on conditions suitable for complement activation ie: fresh serum and the presence of Mg^{++} and Ca^{++} ions. Complement was activated both via the alternative pathway and by the parasite itself via the classical pathway by parasite bound antibodies. In these conditions, cell adherence occurred predominantly through the interaction of leukocyte C3 receptors with parasite bound C3. In contrast, adherence of cells to exsheathed larvae and to the 48 hour and 72 hour post infective larval stages appeared to involve antibody/Fc receptor as well as C3/C3 receptor interactions. The data indicated that N. dubius may undergo a series of antigenic changes during it's life cycle and that antibodies capable of mediating granulocyte adherence are elicited predominantly against the early tissues developmental forms of the parasite.

Granulocytes were further examined for their capacity to damage exsheathed third stage larvae in-vitro. In the presence

of fresh immune serum, both eosinophils and neutrophils caused
a significant reduction in larval infectivity, whereas lymph-
ocytes/monocytes prepared from the same blood samples were
inactive. Neutrophils were as active as eosinophils, on a cell
for cell basis. None of the cells exhibited larvicidal activity
in the absence of serum and serum alone had no effect. In
addition, neutrophils and eosinophils caused a reduction in
larval infectivity in the presence of normal serum ie: via altern-
ative pathway activation and adherence of cells via C3/C3
receptor interaction. However, in contrast to neutrophils,
eosinophils were considerably less effective in normal serum than
in immune serum. Both immune and normal serum were ineffective
if they had been heat inactivated or incubated with methylamine
to destroy complement which indicated that in-vitro antibodies
were incapable of directing the activity of either cell in the
absence of complement.

Of interest was the finding that neutrophils from uninfected
mice, were unable to reduce larval infectivity in the presence
of fresh normal or immune serum. "Altered" neutrophils possessing
larvicidal activity appeared in the blood of mice within 4 days
of infection.

To further assess the role of granulocytes in resistance to
N. dubius in-vivo, experiments were carried out, which showed
that mice, which were rechallenged 4 days after a primary infect-
ion of N. dubius larvae, showed an enhanced resistance to the
second challenge infection, provided immune serum was also
transferred within 48 hours of the second challenge. This
increased resistance was attributed to the action of stimulated
neutrophils, along with specific antibody (either of the IgM or
the IgG immunoglobulin class) since activated macrophages were not
present at this early stage of infection. Furthermore, transfer of
neutrophils, but not eosinophils along with immune serum conferred

partial resistance to challenge with N. dubius. The data suggested, that neutrophils were involved in the initial phases of infection and that the cells act in conjunction with specific antibody in providing resistance.

Experiments in-vivo were carried out where monoclonal antibodies against murine granulocytes were transferred to mice to deplete either eosinophils or neutrophils. These experiments further confirmed the effector role of neutrophils in the initial stages of infection. Treatment of mice with anti-neutrophil antibody 4 days after a primary infection of larvae, completely abolished resistance to rechallenge by N. dubius, whereas depletion of neutrophils, 10 days after a primary infection only partially abolished resistance to rechallenge. Depletion of neutrophils or eosinophils, 10 days after a secondary challenge infection of N. dubius, had no effect on resistance to rechallenge. Neutrophils recovered from mice treated with monoclonal anti-neutrophil serum, were shown to be inactive against larvae in-vitro, when compared to neutrophils from unsuppressed mice. This would suggest that stimulated neutrophils, may be preferentially depleted by this monoclonal antibody, and that resistance to reinfection seen in secondary immunised mice treated with the antibody, may be attributed to activated macrophages and/or eosinophils.

The overall data indicates that neutrophils in a stimulated stage, along with specific antibodies are involved in resistance during the early stages of infection by N. dubius. Activated macrophages and possibly eosinophils are involved in resistance during the later stages of infection.

STATEMENT

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ACKNOWLEDGEMENTS

ABBREVIATIONS USED IN THIS THESIS

EDTA   ethylenediaminetetraacetate

EGTA   ethyleneglycol-bis (Beta-aminoethyl) N,N'-tetraacetate

ELISA   Enzyme-linked immuno-sorbent assay

Ig   Immunoglobulin

IMS   Immune mouse serum

L3   Third stage (infective) N. dubius larvae

N.D.   Not done

NMS   Normal mouse serum

tris   Tris (hydroxymethyl) aminomethane

VB^{2+} Veronal buffered saline supplemented with Ca^{++} and Mg^{++} ions

TO

MUM AND DAD

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CHAPTER 1.

INTRODUCTION

1.1   GENERAL INTRODUCTION

Parasitic helminth infections in the tropical and sub-tropical countries continue to be of major health importance, because of their high incidence and the effect they have on the population, and economic status of the countries affected. Disease caused by helminth infection is likely to produce a chronic debilitating condition in which symptoms develop gradually. However, rarely does the disease result in a high mortality rate. Furthermore in parasitised hosts, particularly those in the poorer rural areas it is not uncommon for polyparasitism to occur, infection with Ascaris, Trichuris and Hookworm being a common combination found in endemic areas (W.H.O. Technical Report, No. 666). Poor diet and subsequent malnutrition further lower the resistance of populations to other infections resulting in serious manpower and economic losses among rural populations. Therefore the importance of helminth infection in human and animal health cannot be ignored, particularly in the developing countries in which the most prevalent incidence of infection occurs.

Stoll, (1947) assessed the number of human cases of Nematode infection at that time to be approximately 1,700 million out of a total world population of 2,000 million. Hookworm, Ancylostoma duodenale and Necator americanus were estimated to infect 456 million people. With the increase in the world population since 1947 these estimates are likely to be extremely conservative, even though effective anti-helminthic drugs are now available. Anti-helminthic drugs may eliminate nematodes from infected hosts, however reinfection usually occurs quickly once people return to infested areas, making control of the incidence of reinfection extremely difficult (Gilles et al., 1964). Progress at the present time in the management of helminth infections has been minimal in developing countries.

In the Ilanos of Venezuela for instance, the prevalence of
Hookworm within the rural population in 1926 was 69%. Between
1950 and 1958, 58% of the population were infected, whereas
recently in the 1970's, 40% of the rural population were
positive for Hookworm, yet the absolute number of infected
subjects has shown no signficant reduction (W.H.O Technical
Report, No. 666).

Eradication of disease caused by helminths is not a
realistic goal in most countries at the present time. Available
techniques such as repeated treatment of infected individuals
with anti-helminthic drugs as in the case of Hookworm, are
only aimed at reducing the load of infection below the level
of clinical significance as reinfection is almost certain to
occur. The success or failure of programs for the eradication
or control of the incidence of infection depends largely on
education programs to teach improved sanitation, changes in
diet and cooking procedures and other factors to minimise the
incidence of infection. Success also depends on community
participation in these programs. Ultimately the development
of suitable vaccines which stimulate that arm of the host
immune response, which is most effective against the parasite
would be a more satisfactory means of controlling helminth
infections, along with such programs as above, within the
community.

1.2   VACCINATION AGAINST HELMINTHS

Some degree of acquired resistance to reinfection by
helminths does occur over time, and it is on the immune
mechanisms involved in such acquired protective immunity that
the prospect of the development of vaccines is based.
Immunisation against helminths infecting man and domestic
animals would be of major health importance and considerable
economic advantage.

However despite the fact that effective vaccines are available for the control of many bacterial and viral infections, the immunological control of helminth infection has yet to be attained.

In general, irradiated - attenuated live larval forms of helminths, somatic extracts of helminths, and secretory/excretory antigens produced by helminths have been considered for use as vaccines. Irradiated - attenuated larvae used for vaccination must be capable of surviving, migrating and producing functional antigens, but have a decreased pathogenicity (Lloyd, 1981). This approach to immunisation against helminths has the dis-advantage in that irradiated larvae have the potential to survive and mature within the host. With nematodes some protection against infection has been achieved by immunisation with attenuated - irradiated larvae. For example, protection against infection by Haemonchus contortus may result from immunisation of adult sheep with larvae treated in this manner (Urquhart et al., 1966). Similarly irradiated larvae of Trichostrongylus colu briformis were highly efficient immunogens in sheep older than 5 months of age (Gregg et al., 1978). Unfortunately young lambs cannot be immunised in this way as they do not respond with a protective immune response. In the field within endemic areas, it is the young lambs which are subjected to severe parasitism, therefore limiting the usefullness of the vaccine. Irradiated forms of Schistosoma species have been used as immunogens to vaccinate animals against infection (Taylor, 1980). Irradiated forms of S. mattheei and S. bovis given sub-cutaneously to sheep or cattle resulted in reduced levels of infection upon a second challenge. Immunisation of sheep with S. mattheei gave a 60% reduction in the level of infection after challenge, even when reinfection was delayed up to 55 weeks (Bickle et al., 1979).

Even though protection in the field was not absolute, some economic advantage within endemic areas could result from such a vaccine as immunised animals have increased growth rates, higher body weight and less histo-pathological symptoms of infection.

Only limited success has been achieved with vaccines using killed helminths or crude somatic extracts of helminths as immunogens (Clegg and Smith, 1978). Secreted antigens for some helminths, such as the cestodes on the other hand have proved to be highly effective immunogens, and induce protection against reinfection. A protective immunity against Taenia ovis and T. taeniaeformis was induced in lambs and rats following exposure to oncospheres grown in millipore chambers implanted into the peritoneal cavity (Rickard and Bell, 1971a). In addition excretory/secretory antigens collected from in-vitro culture of oncospheres were effective in the immunisation of sheep against T. ovis, or cattle against T. hydatigena and T. saginata (Rickard and Bell, 1971b, Rickard et al., 1982).

Secreted antigens have also been used for immunisation against nematodes. In this case, it appears that antigens associated with moulting are the most important in conferring protection (Stromberg, 1977). It was shown that only moulting fluid of Ascaris suum produced during the moult from the third to the fourth stage larvae induced significant protection, other secreted antigens were ineffective. Unfortunately with most other nematodes and cestodes, vaccination with secreted antigens has produced variable results, most proving to be ineffective as reviewed by Clegg and Smith, (1978).

For vaccines against helminths to remain a viable possibility such factors as the effect of different routes of immunisation on the development of protective immunity, the need for, and the type of adjuvant required, the effectiveness

of a complex mixture of antigens compared with highly purified antigens, when the latter are difficult to obtain must be considered. In addition, the isolation and characterisation of antigens leading to a protective immune response must be achieved. Some antigens have been isolated and at least partially characterised (Kwa and Liew, 1977; Stromberg, 1979). The need for isolated antigens would be of particular importance where no in-vitro culture system exists for the production of large quantities of helminth material. Genetic engineering and cloning techniques may be of use in such cases to produce defined antigens in large quantities.

1.3   THE IMMUNE RESPONSE TO HELMINTHS

Resistance to helminth infection involves a complex interplay between cellular and humoral factors. The size and complexity of helminths present a large array of antigens, some stage specific, others common to other parasites, and some which are common to host antigens (Capron and Capron, 1980). The immune response of a host may be directed against the larval or the adult stages of the helminth life cycle. Adults may be expelled from the intestine or destroyed in the tissue, egg production or larval migration may be affected by the immune response.

Reports in the literature have begun to outline the importance of cellular reactions during helminth infections. Increases in the number of mast cells, polymorphonuclear cells, macrophages and lymphocytes have been reported as a consequence of infection, (Kelly and Ogilvie, 1972; Rothwell and Love, 1974; and Prowse, Ey and Jenkin, 1978a). In an immune host it is unlikely that a single cell type would function unaided as an effector against a given helminth parasite, thus a number of effector mechanisms probably act in concert to establish resistance. However, a study of the relative role of individual cell types which may be involved in resistance to

helminths may lead to a clearer picture of the overall immune effector mechanisms. Such a study of the immune effector mechanisms may eventually lead to the development of vaccines that will elicit that response which is most efficient at destroying the parasite.

The following discusses the role of polymorphonuclear cells as effector cells in immunity to helminth infections.

1.4   EOSINOPHILIA DURING HELMINTH INFECTIONS

Many helminth infections in mammals are characterised by high levels of eosinophils in parasitised tissue and blood. Eosinophils originate in the bone marrow and are released into the circulation of chemotactic stimuli, such as histamine release, (Clark, Gallin and Kaplin, 1975), complement activation and the release of C5a, and also by T lymphocyte drived soluable factors which act directly on bone marrow stem cells (Beeson and Bass, 1977; Miller, Colley and McGarry, 1976). The eosinophil is predominately a tissue cell and therefore levels of circulating eosinophils reflect a requirement at a particular tissue site.

Originally the function of the eosinophil was thought to be that of a "biological mop" to prevent the products of anaphylactic reactions leaving localised sites. Eosinophils were shown to have a tendency to accumulate around the site of anaphylaxis possibly due to the release of chemotactic factors during such reactions. Eosinophils also contain enzymes capable of neutralising products of mast cell activation such as histamine and 5 - hydroxytryptamine (Archers, 1963; Kay, Stechschulte and Austen 1971; Gortzl, 1980). Butterworth et al., (1975) were the first to suggest a more aggressive role for the eosinophil during helminth infections by demonstrating that eosinophils were capable in-vitro of mediating antibody dependent damage to the Schistosomula of Schistosoma mansoni.

A passive role for the eosinophil during helminth infection could no longer therefore be credited, since then the eosinophil has been implicated as an effector cell in immunity to a number of helminth parasites (McLaren, Mackenzie and Ramalho-Pinto 1977; Vadas et al., 1979; Ackerman et al., 1981; Haque et al., 1982). Furthermore, since eosinophilia is considered to be under T lymphocyte control, it would imply that the appearance of eosinophils as a consequence of helminth infections was associated with a direct immune attack against the parasite, and not simply a passive response to chemotactic stimuli associated with hypersensitivity reactions around the invading parasite (Basten, Boyer and Beeson, 1970; Colley, 1975). Helminth infections may then result in a unique stimulus for T lymphocytes which induces a T cell dependent eosinophilia, since other stimuli which activate T lymphocytes rarely induce an eosinophilia (Ogilvie, 1980).

In some instances the eosinophil response elicited during helminth infections, may be more of a hinderance than a help. Prolonged eosinophilia can result in damage to host tissue such as the heart and nervous system (Iva et al., 1967; Andy et al., 1977; Gleich et al., 1979). The question however, still remains as to what role the eosinophil plays during such infections. The following discusses the role of eosinophils in immunity to two helminth parasites, Trichinella spiralis and Schistosoma mansoni. Although the effect of eosinophils on other helminths have been reported (Green , Taylor and Alkawa, 1981; Haque et al., 1981), the greater proportion of the published literature is concerned with these two parasites.

1.5   THE ROLE OF EOSINOPHILS IN IMMUNITY TO TRICHINELLA
         SPIRALIS

T. spiralis is a gastrointestinal nematode parasite of man, and rodents   Infection occurs as a result of the

ingestion of meat containing encysted larvae. The acidity
of the stomach releases the larvae from the cysts, and they
then enter the intestine and undergo two moults to reach
maturity. Adult females then deposit eggs in the mucosa, which
hatch approximately 5 days post - infection. The newborn worms
migrate through blood vessels and lymphatics to striated muscle
cells where they encyst to become muscle stage larvae, and may
remain alive for a number of years. After copulation the male
adult dies, the females may persist for several weeks,
depending on the species, but are eventually expelled from the
small intestine.

In human Trichinosis, recovery from infection usually
occurs, unless the initial infection is unusually heavy. The
pathological effects of infection are associated with the
migration of larvae through muscle tissue and their encystment.
Host resistance is predominately directed against newborn larvae
which penetrate the intestinal mucosa and pass via the blood
stream into muscle tissue, or against the encysted larvae
within muscle cells.

For laboratory study the life cycle of T. spiralis can be
reproduced in rodents, making analysis of the immune effector
mechanisms involved in resistance to infection possible.
Infection of rats by T. spiralis, as with other helminths results
in an eosinophil response as characterised by Basten, Boyer and
Beeson,(1970). In rats, natural infection results in the
appearance of eosinophils by day 6, corresponding to the time
that the larvae are deposited in the intestinal mucosa.
Thymectomy or irradiation significantly reduces the eosinophil
response, high-lighting the T lymphocyte dependency of the
eosinophilia. Depletion of eosinophils in mice infected with
T. spiralis results in no apparent effect on the rate of natural
expulsion of adult worms from the intestine.

However, muscle stage larvae double in number, implicating the eosinophil in immunity to the larval stages, but not to the adult stages of T. spiralis (Grove et al., 1977).

It has been shown in-vitro that eosinophils adhere to and cover the entire surface of infective larvae and adult worms in the presence of fresh normal serum, suggesting that complement alone is sufficient to promote adhesion. In contrast, newborn larvae activate complement by the alternative pathway, in a small area in the mid section of the worm, adherent cells being detected only in this area of the cuticle (Mackenzie, et al., 1980) Adherence of eosinophils could also be mediated by specific antibody on the parasite surface binding to Fc receptors on the eosinophil surface. The antibody in humans promoting adherence to newborn larvae has been shown to be of the IgG class, these antibodies arise within three weeks of infection, and are distinct from the IgG and IgM antibodies, which also develop during this acute phase of infection, but mediate adherence to muscle stage larvae only (Kazura, 1981).

Adult worms were not killed by adherent eosinophils, although damage to the parasite cuticle was detected. In contrast, both infective and newborn larvae can be killed by adherent eosinophils in the presence of fresh immune serum. Although eosinophils could adhere to the larval surface in the presence of fresh normal serum, no killing by the eosinophils resulted, specific antibody being required to initiate killing by these cells. The inability of eosinophils to kill in the presence of complement alone contrasts with the observations made with trematode parasites, where these cells have been demonstrated to kill Schistosomula in the presence of normal serum (Ramalho-Pinto et al., 1978). Rats mount a stage specific antibody response to infection by T. spiralis, which can be detected by an antibody mediated eosinophil adherence assay

(MacKenzie, Preston and Ogilvie, 1978; Bell, McGregor and Despommier, 1979). In addition the host protective immune response to T. spiralis has also been shown to be stage specific by an antibody dependent cell cytotoxicity assay, where it was shown that immunity correlated with the appearance of these stage specific antibodies mediating eosinophil cytotoxicity, (Kazura and Grove, 1978). The specificity of the antibody response to each stage in the life cycle was precise, in that antibodies which recognised the surface antigens of one stage did not recognise those of another stage (Philipp et al., 1981). The association of stage specific antibody capable of mediating eosinophil adherence and the stage specificity of host protection suggests a role for the eosinophil and specific antibody in immunity.

Ultrastructural studies of the interaction of eosinophils and the larval stages of T. spiralis show that eosinophils adhere rapidly to the surface of newborn and infective larvae in the presence of fresh immune serum (Kazura and Grove, 1980). The cells degranulate and deposit a dense material onto the parasite surface. As a result, holes and gaps appear in the cuticle and death occurs. The mechanisms by means of which eosinophils kill the parasite and penetrate their surface cuticle will be discussed later.

1.6   THE ROLE OF EOSINOPHILS IN IMMUNITY TO
         SCHISTOSOMA MANSONI.

S. mansoni is a trematode parasite which can infect man, primates and rodents. Infective larvae, cercariae enter the blood stream by burrowing through the skin. After moulting to become schistosomula, they migrate to the lungs via the pulmonary vessels where they burrow through the alveoli causing extensive damage. From the thoracic cavity the schistosomula enter the liver and eventually the mesenteric veins where

copulation and egg laying occurs 22-23 days after infection.
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 tissue to be passed out with the faeces, hatch
and develop into miracidia. These multiply asexually in the
snail, and emerge as infective cercariae. The pathological
effects of infection are associated with the migration of the
larval stages within the host, and also the deposition of eggs
in the tissue of the liver and intestine with subsequent
granulomatous lesions developing.

During the course of infection, a striking eosinophilia
develops within the host, and the interaction in-vitro of
eosinophils and schistosomula of S. mansoni in the presence of
anti-schistosomula antibodies, was the first demonstration of
an active role of these cells against helminth parasites
(Butterworth et al., 1975). Since then, eosinophils have been
reported to kill schistosomula via both antibody and complement
dependent mechanisms (Ramalho-Pinto, McLaren and Smithers, 1978;
Anwar, Smithers and Kay 1979; Vadas et al., 1980). Human
eosinophils are reported to be more efficient at killing
schistosomula in the presence of antibody and complement, than
in the presence of either complement alone or antibody alone
(Anwar and Kay, 1978). Schistosomula incubated in normal rat
serum became more susceptible to damage by eosinophils than those
coated with purified anti-schistosomula IgG immunoglobulin
(Ramalho-Pinto, McLaren and Smithers, 1978). Killing of antibody
coated schistosomula was enhanced by the addition of complement
components, particularly C3. Schistosomula coated with antibody
alone, or antibody in the presence of purified C1 and C4, or C1,
C4 and C2 were all equally susceptible to killing by eosinophils,
however if purified C3 was added, a significant increase in the
percentage killing by eosinophils resulted (Anwar, Smithers and

Kay, 1979). Damage to schistosomula was more severe and occured within a shorter period of time when eosinophils bound to the parasite surface via C3 - C3 receptor interaction rather than via Fc - Fc receptor interaction, (McLaren et al., 1978).

The eosinophil mediated killing via the interaction with complement component C3 at the parasite surface may be of importance as a natural defence mechanism in a naive host whereby invading schistosomula activate the alternative pathway of complement, releasing chemotactic factors, which then attract eosinophils into the area of parasite invasion. In a resistant host, the combination of antibody and complement could result in a more efficient interaction leading to severe damage of the cuticle of the schistosomula. Only low levels of antibody are required for the in-vitro killing of schistosomula by eosinophils, provided complement is present (McLaren, Ramalho-Pinto and Smithers, 1978). A ^{1}/10,000 dilution of rat anti-schistosomula serum in the presence of eosinophils resulted in 100% mortality of schistosomula in-vitro, provided a ^{1}/200 dilution of normal rat serum was also added. A ^{1}/10,000 dilution of immune serum alone in the assay resulted in only 30-40% mortality.

Chemotactic agents such as complement component C5a, referred to as the eosinophil chemotactic factor of anaphylaxis, have been shown to increase the expression of eosinophil receptors for C3b. Such eosinophils, after exposure to chemotactic agents were shown to have an increased capacity to bind and kill schistosomula, (Anwar and Kay, 1978; Anwar et al., 1980).

1.7   MECHANISMS BY MEANS OF WHICH EOSINOPHILS KILL
         HELMINTHS

Eosinophils can be considered, at least in-vitro to be

effector cells in immunity to helminth parasites, such as T. spiralis and S. mansoni, however the mechanisms whereby eosinophils kill helminths is, as yet unclear. A tight association between the eosinophil and it's target is thought to be required, with the release of granule contents directly onto the parasite surface (Kareen et al., 1980).

A two stage mechanism of adherence was postulated by Vadas et al.,(1980), and Capron and Capron, (1980). In the former case, it was proposed that eosinophils in the presence of specific antibody alone have an Fc - Fc receptor dependent interaction at the parasite surface, along with a temperature dependent irreversible step to ensure greater adherence when compared with other cells such as neutrophils and macrophages. Capron and Capron,(1980) proposed a two signal mechanism of eosinophil adherence. "One signal being provided by the binding of IgG antibody to the Fc receptor on the eosinophil surface. The second signal was provided by the interaction of rat IgG_{2a} antibody binding to mast cell surfaces, and the subsequent release of mast cell products". The mast cell products were thought to increase the IgG mediated eosinophil cytotoxicity. Both mechanisms allowing for the tight association of eosinophils to their target.

Glauert et al., (1978) reported in detail, the events following eosinophil adherence to schistosomula. Eosinophils adhere to the parasite surface, flatten and then discharge their granule contents into the extracellular space between cell and schistosomula. Structural changes occurred in the parasite membrane starting with vacuolation of the tegument, which led finally to it's removal. Eosinophils then attached themselves to the exposed muscle where phagocytosis of the muscle tissue and cellular components occurred, leading to the death of the parasite.

Eosinophils after adherence flatten down onto their target, providing antibody and/or complement are present (Kareen et al., 1980). In the absence of antibody, the cells which adhered, did not flatten, but remained rounded. Major Basic Protein (MBP) is a unique component of eosinophil granules, accounting for up to 50% of the granule contents (Gleich et al., 1974). Purified MBP can bind to, and damage schistosomula in the absence of cells, and antibody dependent eosinophil adherence has been shown to result in the release of MBP onto the parasite surface, leading to damage of the tegument in the area directly under the adherent cells (Butterworth et al., 1979). Damage by MBP is non-specific and would therefore account for the need for a tight association between the eosinophil and it's target, if damage to host tissue is to be minimised.

Particles coated with IgG and C3 can induce eosinophils to secrete their granule contents which include Major Basic Protein, Cationic proteins and Peroxidase (Spry, 1978). Human eosinophil peroxidase has been shown to have anti-helminthic properties against new born larvae of T. spiralis in-vitro. The combination of eosinophil peroxidase on the larval surface and H_2O_2 generation by these cells, resulted in the production of hypochlorous acid and subsequent larval death (Bays et al., 1981).

In addition, recent studies have proposed that circulating eosinophils may be in an "activated" state in patients with an ongoing eosinophilia (Winquist et al., 1982). Patients with an ongoing eosinophilia as a consequence of S. mansoni infection have circulating eosinophils with an increased ability to kill schistosomula in-vitro in the presence of specific antibody (David et al., 1980). Eosinophils from such patients band at a lower density after density gradient centrifugation, and have an increased number of IgG, Fc and C3 receptors and a higher oxygen

consumption rate than eosinophils from normal individuals.
Activation of eosinophils may be another mechanism by which
these cells can act as effectors against helminths. Increased
larvicidal activity of human eosinophils has been reported to
occur as a result of their exposure to soluble factor(s)
released by schistosomula of S. mansoni (Auriault et al., 1982).
Exposure of eosinophils to colony stimulating factors, such as
placental conditioned media also results in an enhanced
cytotoxicity of these cells against antibody and complement
coated schistosomula (Dessein et al., 1982).

In conclusion, the eosinophil, both in-vitro and in-vivo
appears to be well equipped as an effector cell against helminth
parasites, it contains a unique protein, Major Basic Protein
which can directly damage helminths, along with a variety of
other toxic proteins. In addition, the eosinophil may undergo
an activation step in the host such that it has an increased
capacity to kill invading helminth parasites.

1.8   THE ROLE OF NEUTROPHILS IN IMMUNITY TO HELMINTHS

Although eosinophils appear to be well equipped as effector
cells against helminths, they cannot necessarily be considered,
together with antibody and complement, as the sole components
involved in immunity. Neutrophils may also increase in number
as a consequence of helminth infection, especially in the early
phases of the disease (Beeson and Bass, 1977), and mononuclear
cells are also common components in the cellular response at
tissue sites of infection (Henson et al., 1979). All three
cell types have been reported to kill a variety of helminths via
mechanisms dependent on antibody and/or complement mediated
adherence (Green et al., 1981; Anwar, Smithers and Kay, 1979).

Neutrophils have an important role in host defence against
invasion by micro-organisms, however it has only been recently
demonstrated that these cells also have anti-helminthic properties.

When comparing polymorphonuclear cells as effectors against
helminths, it appears that the eosinophil is far more efficient
at causing parasite damage and death than the neutrophil (Anwar,
Smithers and Kay, 1979). This contrasts with the experiments
reported, where both types of granulocytes were compared in
their ability to phagocytose and kill bacteria. Although
eosinophils have been found capable of ingesting and killing
bacteria such as Escherichia coli and Staphylococcus aureus
they have generally been found to be less efficient than the
neutrophil (Cline, Hanifin and Lehrer, 1968). This has usually
been attributed to a lower rate of phagocytosis by eosinophils.
Neutrophils have also been reported to have a higher density of
Fc receptors on their surface, which could account for the
higher phagocytic rate of neutrophils. (Van Epps and Bankhurst,
1978). Eosinophilia during helminth infections is under the
control of T lymphocytes, which contrasts with the increase of
numbers of neutrophils, which can occur in the absence of T cells.
Also factors mediating adherence to and subsequent killing of
helminths by eosinophils and neutrophils differ slightly.
Eosinophils require antibody and/or complement for killing,
whereas neutrophils have no anti-helminthic properties in the
absence of complement. Antibody alone is not sufficient to
induce neutrophil mediated killing of helminths, although the
cells will adhere to the parasite surface.

In earlier experiments the neutrophil was considered to
be a poor effector cell against helminths. This conclusion was
based on experiments comparing neutrophil and eosinophil killing
of schistosomula in-vitro in the presence of antibody alone
(Vadas et al., 1980). Neutrophils were shown to adhere to the
parasite surface, but were unable to cause damage to the cuticle.
However, further experiments demonstrated that neutrophils were
efficient effector cells against schistosomula, provided antibody

and complement or complement alone were present in the assay system (Incani and McLaren, 1981; Dean, Wistar and Murrell, 1974). Phagocytosis of bacteria by neutrophils requires the co-operation of both the Fc and the C3b receptor on the cell surface. It has been proposed that the C3b receptor on the neutrophil initially facilitates the adherence of an IgG - C3b coated particle, ie: recognition of the target. Secondly, the C3b receptor then facilitates the IgG - Fc receptor contact by either a structural rearrangement, or removal of C3b from the Fc region of the IgG molecule, and this then leads to phagocytosis. (Hakansson and Venge, 1982). Therefore dual binding to both the Fc and the C3b receptor may be also required for anti-helminthic activity of neutrophils.

Incani and McLaren, (1981) showed further the requirement of both antibody and complement in the killing of schistosomula by rat neutrophils in-vitro. Absence of killing in-vitro in the presence of antibody alone was attributed to the lack of chemotactic stimuli to attract neutrophils. Addition of a chemotactic agent such as Human Serum Albumin was shown to increase the mortality rate by 30%. This explanation requires more thought, in that cells and larvae were in contact for 20 hours in a very small volume which would theoretically allow for sufficient contact of cells and schistosomula. Further studies have shown that in the presence of antibody alone, neutrophils observed under the electron microscope preferentially phagocytose antigen/antibody complexes at the schistosomula surface, rather than to attack the parasite directly, which could account for the lack of effective killing under these conditions (Incani and McLaren, 1983).

Eosinophils may be more efficient effectors against some helminths, as adherence by these cells is considered to be irreversible when compared to neutrophil adherence under the

same conditions (Vadas et al., 1980; Incani and McLaren, 1981; Mackenzie et al., 1981). In studies by Incani and McLaren (1983), adherent neutrophils unlike eosinophils did not flatten to the surface of the larvae of S. mansoni and T. spiralis. Furthermore, no peroxide positive secretions could be detected at the parasite surface when adherence was mediated by antibody alone, which contrasts to their finding when adherence took place in the presence of antibody and complement.

Complement mediated damage to schistosomula by neutrophils, unlike that of antibody mediated adherence is characterised by the vacuolation of the tegumental membrane and the eventual development of lesions in the cuticle. Neutrophils were seen to remove the damaged tegument and subsequently adhere to the muscle cells of the parasite. Damage was seen to occur at a faster rate when both antibody and complement were present together, than in the presence of complement alone.

Neutrophils contain a wide range of hydrolytic enzymes capable of inducing parasite damage including myeloperoxidase lactoferrin, cationic proteins and toxic oxygen products (Greenwalt and Jamieson, 1977). Cationic protein can damage schistosomula in-vitro (McLaren et al., 1981). Nitroblue tetrazolium can be used to detect toxic oxygen products such as H_2O_2. "Footprints" of Nitroblue tetrazolium were seen on the surface of T. spiralis and N. brasiliensis during neutrophil adherence, although no direct killing has, as yet, been attributed to the action of H_2O_2 alone (Mackenzie et al., 1981). Thus neutrophils may act as a first line of defence against helminth infection.

1.9   NEMATOSPIROIDES DUBIUS INFECTION IN MICE

1.9.1   LIFE CYCLE

Nematospiroides dubius is a Trichostrongyle nematode parasite of the mouse, first described by Baylis (1926). The life cycle has been comprehensively reported by Bryant, (1973). Eggs are laid in the faeces, which then hatch 36 hours later to give rise to first stage larvae. The larvae undergo a moult at 28-29 hours with a second moult occurring at approximately 4-5 days of age to give rise to ensheathed, nonfeeding infective third stage larvae (L3). After ingestion by the mouse, the L3 exsheath in the stomach within minutes. They then penetrate the intestinal mucosa, and by 24 hours, most if not all the larvae have penetrated. A further moult occurs at 90-96 hours after infection giving rise to the fourth stage larvae, a further moult then occurs at 144-146 hours post-infection. Young worms return to the intestinal lumen by 191 hours, where they burrow deeply into the crypts of Lieberkuhn. Egg production occurs approximately 2 days later.

1.9.2   HISTOPATHOLOGICAL CHANGES IN THE SMALL INTESTINE AND STOMACH FOLLOWING INFECTION

In naive mice following challenge with N. dubius there is an infiltration of the submucosa and muscularis by neutrophils within 12-24 hours post-challenge (Liu, 1965a; Panter 1969a). After 4 days macrophages also infiltrate the muscularis mucosa around the encysted larvae (Baker, 1954). By day 6 or 7, the larvae begin to emerge as young worms, and the vacated cysts become filled with cells, mainly neutrophils, macrophages and a few eosinophils, resulting in the formation of granulomatous lesions (Baker, 1954; Panter, 1969a).   These granulomata almost completely regress by day 20-21, (Liu, 1965a).

Histopathological changes in the small intestine and stomach as a consequence of infection by N. dubius, in mice

which have undergone a primary infection have been reported
by Liu, (1965b), and Jones and Rubin, (1974). After challenge
of sensitised mice, a rapid and marked necrosis of the
intestinal mucosa and musularis occurs within 2 days, and
cellular infiltration by polymorphonuclear cells, plasma cells
and macrophages was reported around encysted larvae, such that
the worms were surrounded by a wall of cells (Liu, 1965a).
Jones and Rubin, (1974) showed further that within 24 hours
after challenge of sensitised mice, an inflammatory response
could also be detected in both the mucosa and submucosa of the
stomach. Discrete foci, containing variable composites of
lymphocytes, eosinophils and macrophages could be detected.
Neutrophils were present in small numbers, but in some instances
they predominated the response against the invading larvae.
The inflammatory response in the stomach of these mice persisted
for 2-3 days, and then declined. Larvae could not normally be
detected in the stomach during the first 2-3 days after challenge,
but when present, they were seen to be encapsulated with
neutrophils attached to their surface. During the first 3 days,
within the intestine of these sensitised mice, rechallenged
with N. dubius, neutrophils again predominated the response
against the larvae. By day 4, macrophages infiltrated the area
around the invading larvae, along with eosinophils which were
reported to increase in number from that time onward (Ba ker,
1954). Eosinophils were present throughout the lesions around
encysted larvae forming a halo, the cells eventually encapsul-
ated the larvae, and either inhibited the emergence of young
worms, or actually destroyed the larvae within the lesions.
After 7 days the vacated cysts, together with the lesions
containing trapped worms contained a mixture of cell types
including macrophages, neutrophils, eosinophils and lymphocytes,
which eventually led to granuloma formation.

Regression of these fibrotic granuloma did not occur until day 73, which contrasted with that seen in naive mice challenged with N. dubius where granuloma resolve within 20-21 days.

Histological studies implicate the eosinophil as one of the main effector cells causing parasite death. However, the initial attack on invading N. dubius larvae appeared to depend on the infiltration of neutrophils into the area. These cells may then serve as a front line of defence against infection, with macrophages and eosinophils acting at a later time.

1.9.3   DEVELOPMENT OF IMMUNITY TO N. dubius

Following a primary infection with N. dubius, mice develop a partial resistance to subsequent challenge, characterised by their ability to kill no more than half of the challenge dose of larvae (Prowse, Ey and Jenkin, 1978). In contrast, mice infected two or more times, develop a high degree of resistance to subsequent challenge infection, such that they can kill up to 95% of the challenge dose of larvae (Panter, 1969b; Bartlett and Ball, 1972; Prowse et al., 1978). Resistance depends on the number of previous infections, the use of a live immunising infection, and also on the strain of mouse used (Van Zandt, 1961; Panter 1969b; Prowse et al., 1979b).

The development of resistance in mice to infection by N. dubius was first described by Van Zandt (1961), who showed that mice developed some resistance to further reinfection after three oral immunising doses of N. dubius larvae. The degree of resistance was later shown to be dependent on the number of larvae within a challenge dose, and also on the number of immunising infections given (Panter, 1967; Panter, 1969b; Bartlett and Ball, 1972; Chaicumpa et al., 1977). Immunity was thought to be induced by live larvae, and not by adult

worms within the intestine (Van Zandt, 1961). This was later confirmed by Panter, (1969a) and also by Bartlett and Ball, (1972), who showed that the life span of adult worms was not reduced during repeated infections with larvae.

Histopathological studies of the small intestine by Liu, (1965b) demonstrated that granuloma formation was more severe during secondary infection, which suggested that granuloma in the second infection resulted from the larvae being killed within the intestinal muscularis by an immune response elicited as a result of the primary infection. In contrast Panter, (1969b) proposed that immunity was associated with an immediate hypersensitivity reaction within the gut, such that larvae from a secondary challenge were prevented from penetrating the intestinal wall. It was demonstrated by Panter, (1969b) that intravenous injection of N. dubius antigen, composed of supernatants from homogenised centrifuged adult worm preparations, into mice which had received multiple infections of N. dubius larvae, produced an active cutaneous anaphylactic (ACA) response. Furthermore, hypersensitivity could be transferred passively to the skin of normal mice by immune serum (Passive Cutaneous Anaphylactic reaction, PCA). Primary immune mice were also shown to respond with an active cutaneous anaphylactic reaction to intra-dermal injection of an unrelated antigen - antibody complex of, horse serum - anti-horse serum, serum. Within these mice, there was also a significant reduction in the number of worms in the intestine after injection with the unrelated antigen-antibody complex. This further supported the suggestion of a role for hypersensitivity reactions in immunity. It was proposed that in immune mice, a fresh challenge of larvae may produce an anaphylactic reaction capable of preventing and removing a large proportion of the larvae before they became established.

Investigations by Jones and Rubin, (1974) supported the concept that larvae were expelled from the intestine prior to penetration. Mice which had been previously immunised with three dozes of 200 larvae orally, when rechallenged, rapidly expelled the larvae from the challenge dose, prior to invasion and maturation within the muscularis mucosa. This was concluded from the observation of fewer granulomata in the small intestine. They suggested that in orally immunised mice, 'hypersensitivity reactions may have pre-empted the need for a cellular mechanism in immunity'. In contrast sub-cutaneously immunised mice showed a more gradual decline in parasite numbers, with a larger worm burden being detected. In addition, larvel development in these mice proceeded more normally. It was claimed that in sub-cutaneously sensitised mice, larvae were killed following penetration of the muscularis mucosa.

Jones, (1974) expanded this concept by demonstrating an increased active cutaneous anaphylactic reaction in orally sensitised mice, when compared to sub-cutaneously sensitised mice. This was determined by comparing the diameter and intensity of skin lesions after 30 minutes, following the intra-dermal injection of supernatants of disrupted, homogenised adult worms or larvae, and the intra-venous injection of Evans Blue. Oral immunisation was thought to result in a higher production of reaginic antibody, when compared to sub-cutaneous immunisation, indicating that orally sensitised mice where capable of eliciting a greater hypersensitivity reaction. The data also suggested that the degree of resistance and type of immune response against the larvae was related to the route of immunisation. Removal of larvae in orally sensitised mice was attributed to a hypersensitivity reaction, whereas in sub-cutaneously sensitised mice, larvae were thought to be killed by a more complex mechanism involving lymphocytes, eosinophils and anti –

N. dubius antibody.

Chaicumpa et al., (1977) however, reported that mice sensitised with larvae either by the oral, sub-cutaneous, intra-venous or the intra-peritoneal route, all developed resistance to subsequent challenge. Furthermore, the mechanism of resistance to re-infection acted on the challenge larvae after they had penetrated the intestinal mucosa and muscularis (Prowse, 1979). Prowse, (1979) also showed that mice immunised orally or intra-venously with 200 larvae, and then challenged orally, had significantly more granulomata than control unchallenged immunised mice. The increased incidence of granulomata corresponded with the number of larvae given in the challenge dose, indicating that larvae had penetrated the intestinal mucosa and muscularis irrespective of the route of immunisation, and the larvae were killed after penetration.

The immune response against N. dubius larvae appears to be T cell dependent. Host T cells, as measured by macrophage inhibition studies, recognised adult worm antigens (Bartlett and Ball, 1974). Protection against challenge by larvae could also be passively transferred to naive mice by thoracic duct lymphocytes from resistant mice, supporting the concept of a role for T cells in immunity, (Chaicumpa, 1973). The T cell dependence of the immune response was later confirmed by Prowse et al., (1978b) who demonstrated that athymic nude mice were unable to produce granuloma in the intestinal wall, and only developed partial resistance to rechallenge after two immunising infections. Following two or more infections, nude mice still maintained high worm burdens, whereas worms were lost from immune control mice. Reconstitution of nude mice with T cells resulted in some elimination of adult worms. Thus a full complement of T cells was required for maximum resistance and also for the elimination of adult worms.

It is clear from this discussion that immunity to N. dubius involves mechanisms other than immediate hypersensitivity reactions, and may require the direct interaction of certain effector cells along with specific antibody.

1.9.4 THE IMMUNOGLOBULIN RESPONSE DURING N. dubius INFECTION

A primary infection with N. dubius results in an immunoglobulin response primarily of the IgG_1 class (Crandall, Crandall, and Franco, 1974). Serum IgG_1 levels increase dramatically as measured by radial immunodiffusion, when compared with the levels of the other immunoglobulin classes, which remained relatively constant, except for a small rise in levels of IgM. Specific antibody was detected within the first 2 weeks of infection using an indirect fluorescent antibody technique, on cryostat sections of adult worms. Upon studying the intestinal response during a secondary infection, it was shown by the immunofluorescence technique, that IgG_1 plasma cells were the most common infiltrating cell. Intestinal washes from these mice contained both IgG_1 and IgA immunoglobulin, but antibody was only detected in the IgG_1 isotype.

In contrast Cypess, Ebersole and Molinari, (1977) reported antibody of the IgA class. In these experiments the levels of the various immunoglobulin classes in intestinal washings, was determined before and after removal of specific antibody. The removal of anti-parasite antibody, both precipitating and non-precipitating, was accomplished by the adsorption of intestinal washings with an excess of supernatants derived from homogenised adult worms. The resulting antigen/antibody complexes were precipitated with an excess of anti-worm serum. Antibody by this means, was detected in the IgG_1 and the IgA class, but not in the IgG_{2a} or IgM class. However, the technique of radial -

immunodiffusion used to detect specific antibody may not be
sensitive enough to detect small differences in the levels of
specific antibody prior to and after adsorption with worm
supernatants. For instance, if only a low level of a given
immunoglobulin class for instance either IgG_{2a}, or IgM were
present within the intestinal washings, and only 2% of the
immunoglobulin in this class were specific antibody, then in the
assay used a difference in specific antibody levels prior to,
and after adsorption would not be detected. The IgG_1 immuno-
globulin associated with intestinal washings may result from the
leakage of serum proteins into the lumen as a consequence of
larval emergence and/or the establishment of the adult worm
population, and not be due to the actual secretion into the lumen.

The levels of serum immunoglobulin were later assessed by
Prowse et al., (1978a) by the use of the more sensitive technique
of radioimmunoassay.   An enormous primary IgG_1 immunoglobulin
response was detected after challenge, which further increased
after a secondary challenge to levels as high as 30-50 mg/ml.
Again no significant changes in the levels of the other
immunoglobulin classes could be detected. Antibody to N. dubius
was always found to be associated with the IgG_1 isotype
(Crandall et al., 1974; Prowse et al., 1978a).

IgG_1 hypergammaglobulinaemia is seen in many helminth
infections in mice, particularly where a chronic infection
results (Chapman et al., 1979a). The response has been shown
to be T cell dependent and may 'reflect chronicity of antigen
exposure' (Chapman et al., 1979b). An attempt was made by the
above investigations to simulate the chronic antigenic exposure
resulting from such infections using repeated immunisations of
high doses of sheep red cells. This procedure also resulted in
a higher number, and higher proportion of IgG_1 plaque forming
cells. It was proposed that the enormous levels of IgG_1 found

during chronic helminth infection 'reflected persistent, high

dose, 'strong' T cell dependent stimulation of the B cell

system', and resulted in a preferential production of IgG_1

immunoglobulin. The immunological significance of the IgG_1

response, however remains unclear.

Attempts in the past to passively transfer immunity to

N. dubius with immune serum have largely proved to be unsuccess-

ful (Panter, 1969b; Chaicumpa et al., 1976; Prowse et al., 1978b).

However, recently a protective role for antibody in-vivo during

N. dubius infection has been proposed by Behnke and Parish

(1979), who showed that passive transfer of immune serum from a

high responder NIH strain of mice, resulted in the expulsion of

adult worms from the intestine. For such an effect, immune serum

had to be transferred to the recipients on, or before day 6 of

infection, suggesting that the immune mechanism operating, acted

on the larval stages during early development.

The NIH strain of mice has been reported to respond

rapidly to several other nematode infections (Wakelin, 1975;

Wakelin and Lloyd, 1976). Activated macrophages have also been

implicated in immunity to N. dubius (Chiacumpa and Jenkin, 1978).

The possibility of the presence of a cell population, presumably

macrophages, in an already activated state within these mice

cannot be excluded, and may account for the ability to transfer

immunity to NIH mice by the passive transfer of serum alone.

Jenkins and Carrington, (1980) assessed resistance to infection

by N. dubius in adult high and low responder Biozzi mice with

the above question in mind. Biozzi mice were claimed to exhibit

differences in their humoral response to a variety of antigens,

furthermore, macrophages from the low responder mice were

reported to be in an 'activated' state, when compared to

macrophages from the high responder mice. After oral immunisation

with N. dubius larvae, no difference in the response to a
primary infection was detected between the high and low
responder strains. However, after exposure to a second or
third infection, high responder mice were shown to be more
resistant to infection, with fewer adult worms present in the
intestine, when compared with low responder mice. Therefore it
was suggested that host antibody played an essential role in
immunity.

The macrophages from the low responder mice were in an
'activated' state, yet the mice were equally susceptible to
primary infection as the high responder strain, indicating that
'activated' macrophages alone are not sufficient to confer
resistance. Low responder mice do develop a high resistance,
but only after a tertiary infection, which suggests that repeated
immunisations were required to result in the production of
sufficient antibody to mediate in the immune response against
N. dubius. Since immunity to N. dubius cannot be attributed
solely to the action of antibody alone, other effector
mechanisms must also be involved as for example, the attack of
polymorphonuclear cells and macrophages on the larvae within the
intestinal mucosa and muscularis.

1.9.5   THE CELLULAR RESPONSE IN IMMUNITY TO N. dubius.

Infection of mice with N. dubius not only resulted in a
huge IgG_1 immunoglobulin response, but also in a change in the
levels of circulating leukocytes (Prowse et al., 1978a). After
primary infection, a five fold increase in total circulating
blood leukocytes may occur. Cell levels peaked at day 6-8 post-
challenge, and then slowly declined, the peak correlating with
the time that young worms began to emerge into the intestine.
Levels of peritoneal leukocytes also increased, and in contrast
to blood leukocyte levels, peritoneal cell numbers were still

elevated at the time of the second challenge (day 14).

Primary infection of mice by N. dubius resulted in a 40-50 fold increase in blood neutrophil and lymphocyte levels, whereas in the peritoneal cavity, macrophages as well as neutrophils and lymphocytes increased in number. Only a 2-4 fold increase in macrophages occurred compared to a greater than 20 fold increase in lymphocytes and neutrophils, however, the absolute number of macrophages was always 3-4 times higher than that of other cell types in the peritoneal cavity. After a primary infection no eosinophils could be detected within the circulation, or the peritoneal cavity, however they were present within the bone marrow. A secondary challenge resulted in a sudden and dramatic appearance of eosinophils 7 days later, which reached levels as high as $2-5 \times 10^6$ cells/ml of blood. These high levels were maintained for a number of weeks after a tertiary immunisation (Prowse et al., 1978a). The appearance of eosinophils corresponded to the time that mice were shown to develop full resistance to rechallenge, implicating these cells in immunity to infection. Encysting larvae were thought, by these investigators to be the stimulus for the release of eosinophils from the bone marrow.

Eosinophilia is a characteristic of many helminth infections (Butterworth et al., 1975), yet the role of these cells in-vivo immunity is unclear. Similarly the role of neutrophils in immunity to helminths is also uncertain. Most studies on the effect of cells on helminth larvae have involved the macrophage. Macrophages have been implicated in immunity to N. dubius, the importance of these cells in immunity being stressed by Chiacumpa and Jenkin (1978), who showed that peritoneal cells from immune mice damaged the larvae of N. dubius in-vitro. The predominant cell type present within the peritoneal washout cell population of immune mice was the macrophage.

Peritoneal cells from normal mice where unable to damage larvae under the same conditions (Chaicumpa and Jenkin, 1978). These results suggested that macrophages were required to be in an 'activated' state for efficient killing to occur. This concept was supported by further experiments involving S. enteritidis 11RX. Challenging mice with this organism is known to activate macrophages (Davies and Kotlarski, 1974). Peritoneal cells from S. enteritidis 11RX infected mice also killed N. dubius larvae in-vitro in the presence of N. dubius immune serum.

However both plastic adherent (predominately macrophages) and non-adherent peritoneal cells from resistant mice have been shown to impair the infectivity of larvae in-vitro in the presence of immune serum (Prowse, 1979). Since non-adherent cells damaged larvae, it would imply that cells other than the macrophage were also involved in immunity.

Eosinophils have been implicated in immunity to N. dubius by Hurley and Vadas, (1983). They showed that BALB/c mice developed resistance to N. dubius, 2-3 weeks after a sub-cutaneous injection of adult worms into the base of the neck. In contrast CBA/H mice failed to develop resistance. Furthermore BALB/c mice, as well as BALB/c (BALB/c x CBA/H) F1 bone marrow chimeras were shown to mount a strong eosinophil response, whereas CBA/H and CBA/H (BALB/c x CBA/H) F1 chimeras only elicited a weak eosinophil response following infection with N. dubius. Resistance to infection was therefore attributed to the strength of the eosinophil response of the mice, which supports the concept that eosinophils are important in resistance to N. dubius.

The present thesis examines the reaction of murine eosinophils and neutrophils with N. dubius larvae in-vitro, and assesses the role of these cells in determining the resistance of mice, to infection by this particular parasite.

CHAPTER 2.

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1.1   EXPERIMENTAL ANIMALS

Outbred LACA mice were used predominately throughout these studies. The mice were raised under specific pathogen free conditions in a closed colony at the University of Adelaide Central Animal House. In some experiments an F1 hybrid between C57/BLxBALB/c mice were used as indicated in the text.

2.1.2   INCUBATION MEDIUM AND DILUENTS

The basic medium used in the following experiments was medium 199 (Flow. Laboratories U.S.A.) containing penicillin and streptomycin sulphate (200 units/ml), and 5 - Fluorocytosine (10 ug/ml). The medium was buffered with 1MNaOH and 10mM (final CONCENTRATION) N- 2 - Hydroxyethyl piperazine - N' - 2 - ethane-sulfonic acid (HEPES; Calbiochem) to pH 7.4 Supplemented medium was basic medium to which had been added 3 ml of 0.03M $NaHCO_3$ / 100ml and heat activated foetal calf serum (FCS) to a final concentration of 5%. The diluent used for the cell adherence assays was Veronal buffered saline (VB^{2+}) an isotonic buffer containing 0.3M NaCl, 5mM Sodium barbitone, 0.15mM $CaCl_2$ and 0.5mM $MgCl_2$. The buffer sometimes contained (0.01M ethylene-diaminetetra - acetic acid (EDTA), or (0.01M) ethyl-eneglycol - bis (beta-amino-ethyl ether) N, N'-tetra-acetic acid (EGTA) as indicated in the text. Hanks balanced salt solution was prepared by adding 5ml of solution A plus 5ml solution B to 90ml of sterile distilled water. Solution A was composed of 160gm NaCl, 8gm KCl, 4 gm $MgSO_4$-$7H_2O$, 2.8g $CaCl_2$ dissolved in 1000ml of distilled water. Solution B contained 3.04g Na_2HPO_4-12 H_2O 1.2g KH_2PO4, 20g glucose and 100ml of 0.4% phenolred in 900ml of distilled water. After mixing solutions A and B in 90ml of water, the medium was buffered to pH 7.4 with 0.15M phosphate buffer.

Finally, foetal calf serum was added to a final concentration of 5%.

2.2   MAINTENANCE OF THE PARASITE NEMATOSPIRODES DUBIUS.

200 - 400 third stage larvae (L3) were adminstered orally to LACA mice. These infected mice were used as a source of eggs over a period of 3-4 months. Wire mesh grids were placed in the bottom of cages over moist filter paper to collect faecal pellets. The pellets were then mixed with distilled water and the slurry passed through a 500 u sieve. After centrifuging the filtrate at 1500g for 5 minutes, the supernatant was discarded and the pellet resuspended in distilled water. The resulting sediment was streaked onto moist Whatman filter paper, lining a large glass tray 30 x 30 x 3 cm. These were then left covered with a glass plate at room temperature under high humidity. After 7 days of culture, the larvae were collected by rinsing the filter paper in the tray with distilled water. The larvae were washed and resuspended in distilled water and maintained at 4°C until used. Third stage larvae kept at this temperature for periods of up to three months, still retained full viability as measured by infectivity. The concentration of L3 in the suspension was determined by counting under a dissecting microscope (x16) the number in an 0.1 ml. aliquot placed onto a perspex grid and covered with a coverslip.

2.3   LARVAL EXSHEATHMENT

To obtain exsheathed L3, mice which had been starved overnight were fed with 10,000 L3 in 0.8 ml of physiological saline. Thirty minutes later the mice were killed by cervical dislocation, the stomach and small intestine were removed, cut open along their entire length and incubated in a petri dish containing 30ml of physiological saline at room temperature for

30 minutes. Intestinal debris was then removed by filtration through a thin layer of cloth. The filtrate containing the larvae was centrifuged at 500 g for 1 minute and the larvae were resuspended to a final concentration of 1,000/ml in 199 Medium (Commonwealth Serum Laboratories) containing penicillin and streptomycin sulphate (each 200 units/ml) and 5 - fluoro-cytosine (10 ug/ml). These exsheathed larvae could be stored for 3-4 days at 4°C without noticeable loss of infectivity.

2.4   COLLECTION OF POST - INFECTIVE LARVAE

To obtain larvae from infected mice at various times after infection, 2,500 L3 suspended in 0.4 ml of distilled water were introduced into the lower part of the oesophagus of each mouse using a blunt 19 gauge needle connected to a tuberculin syringe. Mice were killed by cervical dislocation at 48 hours, 72 hours and 96 hours after infection. The tissue stage larvae were then collected by the method of Ey, Prowse and Jenkin (1981) as follows. The entire length of the small intestine from above the pyloric sphincter to the ileo - caecal valve was removed from the mice, and freed of connective tissue. After tying the ends with surgical thread, each intestine was immersed in 15 - 30 ml. of prewarmed (37°C) medium in a 50 x 13 mm. petri dish and incubated at 37°C for two hours. The medium was composed of a 1 : 4 mixture of medium 199 (Commonwealth Serum Laboratories Melbourne), and physiological saline containing 200 units per ml. penicillin and streptomycin sulphate. Emergent larvae were collected from the medium by pouring the larvae from a number of petri dishes into 100 ml. bottles and allowing the larvae to settle onto the bottom. The supernatant was discarded and the larvae washed in medium 199 3-4 times (Fig. 2.1). Finally the larvae were resuspended to a concentration of 2,500 larvae/ml. in Ca^{2+}/Mg^{2+} supplemented

FIGURE 2.1

Post-infective larvae collected from the intestines of mice infected with N. dubius

(a)   48 hour post-infective larvae

(b)   72 hour post-infective larvae

(c)   96 hour fourth stage larvae

(d)   adult worms

a

b

c

d
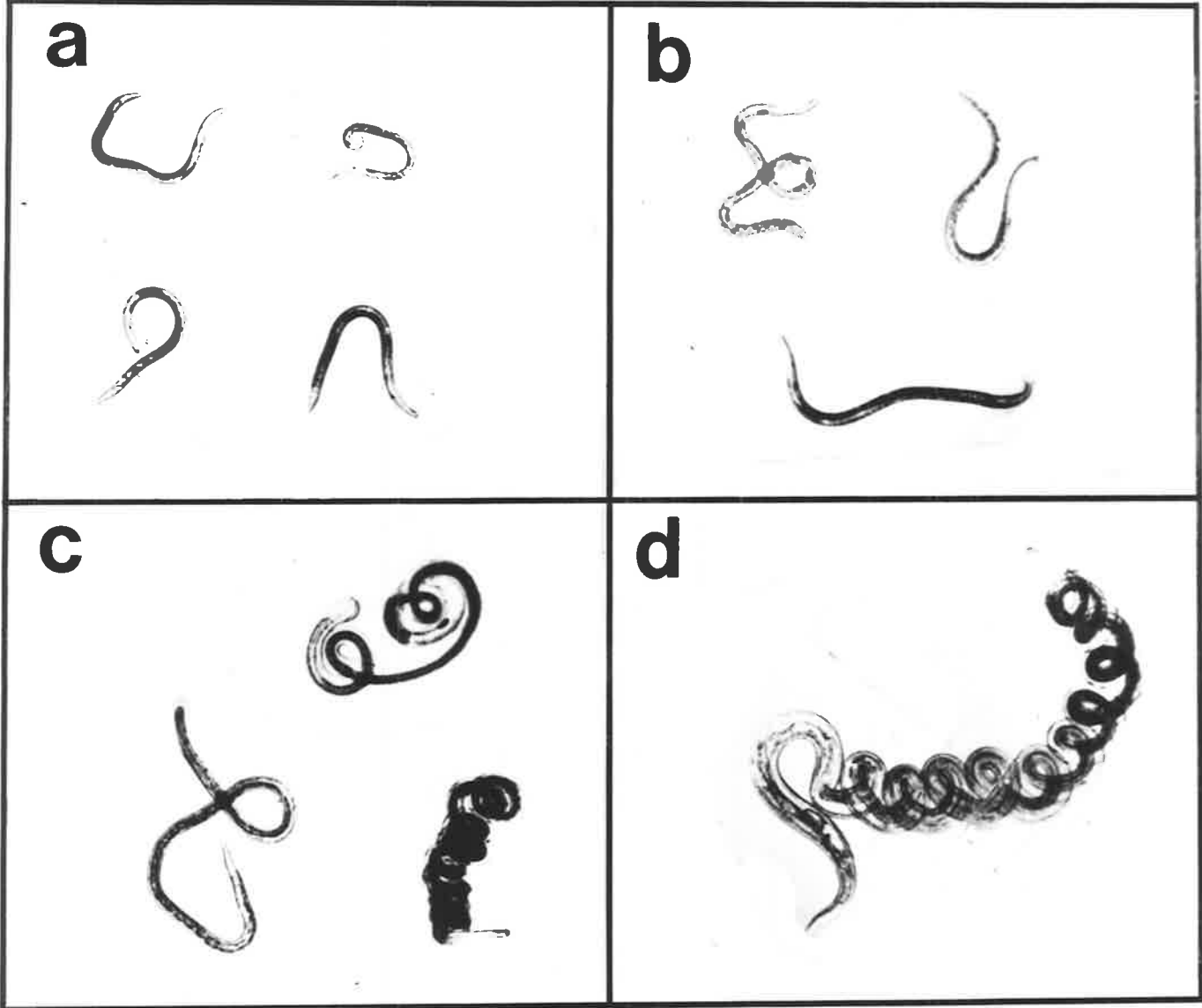
veronal buffer (VB^{2+} : 5mM sodium barbitone, pH 7.4/0.142 M NaCl / 0.15 mM $CaCl_2$ / 0.5 mM $MgCl_2$).

2.5   COLLECTION OF ADULT WORMS

Adult worms were collected from mice 10 days after a primary infection of 200 L3. Mice were killed by cervical dislocation. The entire length of the small intestine to the ileo-caecal valve was removed, freed of connective tissue, and placed into Petri dishes containing 30 ml of physiological saline. The intestine was slit throughout it's entire length and then incubated under a heat lamp for 60 minutes. Under these conditions adult worms migrate out from the intestines into the surrounding medium. Worms were collected using a pasteur pipette and washed in veronal buffer (VB^{2+}).

2.6   PREPARATION OF ADULT WORM EXTRACTS

A group of 100 mice were infected with 400 L3 orally, and 14 days later adult worms collected as above. The worms were frozen and thawed in 90 ml of saline, and then homogenised in an Ultratarax blender and frozen, thawed a second time. To this mixture 0.4 ml of 0.25 M EDTA was then added and the worms re-homogenised and frozen and thawed once more. The homogenate was then centrifuged for 30 minutes at 18,000 g. The supernatant was removed and dialysed overnight against phosphate buffered saline at pH 8.0. Following dialysis the supernatant was stored at -20°C until required.

2.7   IMMUNISATION AND COLLECTION OF SERUM

Mice were immunised by injecting intravenously two to four separate doses of 200 L3 in 0.2 ml of physiological saline at fortnightly intervals. Mice were bled via the retro-orbital plexus fourteen days after the fourth immunisation or ten days after the second. The blood was allowed to clot at room

temperature for 30 minutes, and was then cooled on ice to allow for clot retraction. The immune mouse serum (IMS) was then collected and kept on ice until used in the experiment, which was always within 3-4 hours after bleeding. Normal mouse serum (NMS) was collected from uninfected mice and treated in a similar manner.

2.8   TOTAL AND DIFFERENTIAL LEUKOCYTE COUNTS

Whole blood or purified leukocytes obtained from the peripheral blood were diluted in white cell counting fluid composed of 0.1% gentain violet in 10% v/v acetic acid/distilled water. A haemocytometer was filled with an aliquot of this and the leukocytes counted.

Differential cell counts were performed on cytocentrifuge samples stained with Leishman stain, and at least 200 cells from each slide counted.

2.9   PREPARATION OF POLYMORPHONUCLEAR CELLS

Mice were immunised twice with 200 L3 as described above. The animals were pre-bled via the retro-orbital plexus on day 21 (0.5 ml/mouse) and the blood was discarded. Peripheral blood was again collected three days later from the immune mice into preservative-free lithium-heparin tubes (Johns Professional Products, Australia) and pooled for cell fractionation on a density gradient as previously described (Penttila, O'Keefe and Jenkin, 1982). This resulted in three bands. The top band contained mainly monocytes and lymphocytes. The middle band consisted of 63% $\pm$ 3% eosinophils, 30% $\pm$ 6% lymphocytes and 7% $\pm$ 3% monocytes (mean $\pm$ one standard deviations). The bottom band contained 96% $\pm$ 2% neutrophils, 1% $\pm$ 1% eosinophils and 2% $\pm$ 1% lymphocytes. (See chapter 3).

2.10   PERITONEAL WASHOUT CELL COLLECTION

Peritoneal washout cells were collected in the following manner. Mice were killed by cervical dislocation and the skin over the abdomen deflected. Two ml of ice cold supplemented medium were then injected into the peritoneal cavity. The abdomen was massaged gently and the fluid withdrawn with a 22 gauge needle mounted onto a 2 ml syringe. The cells were then transferred to a siliconised tube on ice and washed twice in supplemented medium, by centrifugation at 500 g for 5 minutes. Finally the cells were resuspended in the above medium. In some experiments Hanks Balanced Salt Solution was used in place of supplemented medium.

2.11   ADHERENCE OF EOSINOPHILS AND NEUTROPHILS TO THE DIFFERENT LARVAL STAGES OF N. dubius

Eosinophils or neutrophils were resuspended at a concentration of 4×10^6 cells/ml in supplemented medium. To siliconised glass tubes containing 0.05 ml of NMS, IMS or VB^{2+} were added 500 worms of the particular stage to be tested, suspended in 0.2 ml of VB^{2+} or VB^{2+} plus either 10 mM EDTA or 10 mM EGTA as indicated. The contents of the tubes were mixed and incubated at 37°C for 90 minutes with gentle agitation. Each suspension was then diluted with 5 ml of supplemented medium and centrifuged at 500 g for 1 minute. The supernatant was removed and the larvae were washed again in supplemented medium. The supernatant was again removed leaving the larval pellet in approximately 0.25 ml of medium.

To the washed larvae were added 0.25 ml of supplemented medium containing 1×10^6 neutrophils or eosinophils. The tubes were incubated for 60 minutes at 37°C, and the adherence of the cells to the larvae was then scored in the following manner. Microscope slides, which had been dipped in 5% Bovine Serum albumin (BSA) and allowed to dry, were used. A drop of the

suspension containing the larvae and cells was placed onto a slide and allowed to partially dry. An equal volume of Leishman's stain was then placed onto the drop of larvae and left for 1 minute. Finally a cover slip was placed on the slide. The number of cells adhering to each of 30 larvae was counted by light microscopy under 1000x magnification and the average number of cells adhering to each worm was calculated.

2.12 IN-VITRO ASSAY FOR MEASURING THE EFFECT OF POLYMORPHONUCLEAR CELLS ON THIRD STAGE LARVAL INFECTIVITY

The assay was carried out in sterile, siliconised 12 ml glass tubes. The medium used throughout was supplemented medium. To 200 exsheathed L3 suspended in 0.2 ml of supplemented medium were added either 0.05 ml of immune mouse serum (IMS), normal mouse serum (NMS) or heat inactivated (56°C, 30 min) immune mouse serum (H-IMS) (5 tubes/group). The larvae and serum were incubated for 90 minutes at 37°C with gentle agitation, following which eosinophils or neutrophils were added in 0.2 ml of supplemented medium. The tubes were then incubated at 37°C for a further 60 minutes. Finally, 5 ml of supplemented medium were added, the tubes were gassed with 5% carbon dioxide in air and incubated at 37°C for 48 hours. At the end of the incubation period, the tubes were centrifuged at 500 g for 1 minute to pellet the larvae and all but 0.5 ml of the supernatant were removed. The contents of each tube were then administered per os to naive mice (1 tube/mouse) to assess the infectivity of the larvae. Encysted larvae in the wall of the small intestine of each mouse were counted 6 days later.

2.13 ASSESSMENT OF IMMUNITY IN MICE TO N. dubius INFECTIONS

Immunity to N. dubius infection was determined by comparing

FIGURE 2.2

Small intestine showing cysts from mice 6 days after
challenge with L3

C = cyst

the number of encysted larvae in immunised mice with the number
in naive mice following oral or intra-venous challenge with
200 L3. For oral immunisation larvae (L3) were introduced into
the lower part of the oesophagus using a tipped 19 gauge needle
connected to a tuberculin syringe. For intravenous challenge
200 L3 in 0.2 ml of saline were injected into a tail vein using
a 25 gauge needle. Cysts were counted on the 6 day after
challenge. The small intestine was removed from below the
pyloric sphincter to the ileo-caecal valve, and pressed between
two pieces of transparent perspex. Both sides at the exposed
intestine were scanned under a dissecting microscope (16x
magnification) and the number of cysts present counted, (Fig.
2.2).

2.14 PREPARATION OF MOUSE IMMUNOGLOBULIN IgM AND IgG FROM THE SERUM OF N. dubius INFECTED MICE BY SEPHADEX G-200 CHROMATOGRAPHY

Forty millilitre aliquots of serum were fractionated at
4°C on a 100 x 5.5 cm column of Sephadex G-200 previously
equilibrated in isotonic Tris buffered saline (tris hydroxymethyl
amino methane, 25 mM, NaCl, 132.5 mM, ethylene diaminetetra
acetic acid, 0.1 mM, NaN_3, 8.3 mM) pH 8.0. Using the same buffer
at a flow rate of 36 ml/hour, 18 ml fractions were collected.
The absorbance of each fraction was measured at 280 nm and/or
295 nm to identify the major protein peaks (macroglobulin, IgG
and albumin). These were pooled separately concentrated by
ultra-filtration and analysed by ELISA for IgM and IgG.

2.15 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

The concentration of immunoglobulin isotypes in serum
fractions after Sephadex G200 chromatography was estimated by
a competitive inhibition ELISA. Soluble immunoglobulin in the
test sample was assessed for ability to inhibit binding of
alkaline phosphatase conjugated to affinity purified rabbit

antibodies, specific for the heavy chain of the appropriate mouse immunoglobulin to wells coated with purified mouse IgM, IgG_1, IgG_{2a}, IgG_{2b} and IgA. The assay was performed in 96 well round bottomed acrylic microtitre trays (Linbro, Flow Laboratories, U.S.A.). The wells were coated with purified mouse immunoglobulin (5 ug/ml) in 25 mM tris-buffered saline, pH 7.5 + 0.1% NaN_3. After 1-2 hours at room temperature, the wells were washed twice with PBS containing 0.05% Tween 20 and 0.2 mg of BSA (washing buffer; 190 ul/well). Eighty micro-litres of washing buffer were then added to each well and the test sera and reference immunoglobulin solution (of known concentration) were each titrated in duplicate in serial 2 - fold dilutions across the rows of the tray. The reference solution was used to construct a standard curve of immunoglobulin concentration. Control rows consisted of uncoated wells, and coated wells containing only washing buffer. To every well was added 100 ul of the appropriate enzyme - antibody conjugate (2 ng of antibody) in 20 mM Tris - HCl / 0.15 M NaCl / 0.1 mM $MgCl_2$ / 0.25 uM $ZnCl_2$ / 0.2 mg/ml BSA/0.1% NaN_3 (pH 7.5). Trays were covered and incubated overnight at room temperature. The wells were then washed twice with washing buffer and finally 160 ul of substrate solution (1 mg/ml of disodium p - nitrophenyl-phospate; Sigma Chemical Company U.S.A.) in 1M diethanolamine - HCl/1mM $MgCl_2$/0.05% NaN_3 (pH 9.8) was added to each well. The trays were incubated at 37°C for 4 hours, after which the amount of p - nitrophenol produced in each well was assessed spectrophotometrically by reading the absorbance at 405 nm.

2.16   PREPARATION OF MOUSE ANTI-SHEEP RED BLOOD CELL
        SERUM, IMMUNOGLOBULIN CLASSES.

100 CBA mice were immunised with 0.2 ml of 2% SRBC intra-venously in saline on day 0 and again on day 20. Mice were bled from the retro-orbital plexus on day 24, and the blood

allowed to clot on ice. Serum was collected, and the haemagglutination titre against a suspension of 1% SRBC determined. A haemagglutionation titre of [1]/3600 was obtained.

The anti-SRBC serum was fractionated by protein - A - Sepharose chromatography as described by Ey, Prowse and Jenkin, (1978). Briefly, 4-5 ml of serum was mixed with 2 ml of 0.1 M sodium phosphate pH 8.2, and applied to the protein - A - Sepharose column which had been equilibrated with 0.1 M sodium phosphate pH 8.2 at 4°C. The column was washed until no more protein was recovered in the effluent as determined by absorbance at 280 nm. This pool contained IgM anti-SRBC anti-bodies, which were further purified by gel filtration of Sephacryl S-300. Pure IgG_1, IgG_{2a} and IgG_{2b} immunoglobulins, which had bound to the column were then recovered by sequential elution with 0.1 M Citrate buffers at pH 6.0, 4.5 and 3.5 respectively. The haemagglutination titre of the anti-SRBC immunoglobulin fractions were then determined against a suspension of 0.05% sheep red cells.

2.17   MONOCLONAL ANTIBODIES FOR THE SELECTIVE DEPLETION
        OF EOSINOPHILS AND NEUTROPHILS FROM N. dubius
        INFECTED MICE

Monoclonal antibodies NIMP-R10 and NIMP-R6 were kindly donated by Dr.A.Lopez of the Walter and Eliza Hall, Institute of Medical Research, Melbourne, Australia. The binding characteristics of these two monoclonal rat IgG_{2a} complement fixing antibodies used in the following experiments, have been reported by Lopez et al., (1983).

NIMP-R10 recognises a differentiation antigen present on mature non-dividing granulocytes and binds to mouse eosinophils, neutrophils and macrophages, being cytotoxic in-vitro in the presence of complement, while NIMP-R6 binds preferentially to mouse eosinophils, and is also cytotoxic in the presence of

complement. Both antibodies were used as clarified ascitic fluid from rats, which had been injected with the corresponding hybridoma cells. To deplete polymorphunuclear cells from mice infected with N. dubius, the antibodies were injected into the mice intraperitoneally (i.p.) either 1 ml of a ^{1}/100 dilution of NIMP-R10 in saline, or a ^{1}/25 dilution of NIMP-R6 was given to mice on days -1, 0 and 1, day 0 being the day of challenge.

2.18   INACTIVATION OF SERUM COMPLEMENT BY METHYLAMINE

In order to assess the role of antibody alone in the in-vitro larvicidal assay with polymorphonuclear cells, serum complement activity was destroyed by methylamine. Samples of fresh immune or normal serum (2 ml) were mixed with 0.2 ml of 1M methylamine HCL/0.13 M Sodium phospate at, pH 8.0 and incubated at 37°C for 90 minutes. The sera were dialysed at room temperature against 13 mM phospate buffered saline (pH 7.4) supplemented with 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$.

2.19   STATISTICS

Data was analysed by the 'U' test of Mann and Whitney. A probability at 0.05% or less was taken as being significantly different.

TECHNIQUE FOR ENRICHING EOSINOPHILS

AND NEUTROPHILS FROM MURINE

PERIPHERAL BLOOD

3.1   INTRODUCTION

For an _in-vitro_ study on the interaction of various cell types with parasites, it is desirable to obtain highly purified, functionally active cell populations. The development of a technique which results in the purification of a given cell type in quantities, which will permit an analysis of their functions, is therefore of considerable importance. Numerous investigators have employed density gradients of varying composition to enrich different cell types. There are several methods reported for the enrichment of human eosinophils, most of them based on this technique (Boyum, 1968; Ferrante and Thong, 1978; Gartner, 1980). The human eosinophil has the highest density of all human leukocytes, and therefore lends itself to this type of separation procedure, thus enabling functional studies to be undertaken. In contrast, functional studies on murine eosinophils have been restricted by difficulties in the preparation of enriched cell populations in sufficient quantities to enable one to carry out such investigations _in-vitro_.

As a consequence of infection of mice with _N. dubius_, as with most helminth infections, there is a marked eosinophilia, (Prowse, Ey and Jenkin, 1978). The appearance of these cells in the circulation corresponds with the time that mice have been shown to develop full resistance to subsequent challenge infections, suggesting a possible effector role for these cells in immunity. For an analysis of the role of eosinophils in immunity to _N. dubius_ it was necessary to develop a suitable technique for enriching these cells from infected mice.

3.2   TOTAL AND DIFFERENTIAL LEUKOCYTE COUNTS ON PERIPHERAL BLOOD AND PERITONEAL WASHOUTS FROM MICE RESISTANT TO N. dubius INFECTIONS

As a consequence of a secondary infection of mice by _N. dubius_ eosinophils can be detected in large numbers in the

circulation and the peritoneal cavity, providing a convenient starting source of cells for enrichment. Prior to experiments designed to enrich eosinophils from infected mice, total and differential leukocyte counts on peripheral blood and peritoneal washouts were carried out on mice infected with N. dubius. A group of mice were given two doses of 200 L3 intravenously, at 14 day intervals, ten days after the second infection blood leukocyte counts were carried out as previously described in Materials and Methods, (Section 2.8).Total leukocyte counts from these mice ranged from $0.5 - 1 \times 10^7$ leukocytes/ml of peripheral blood and $0.5 - 1.2 \times 10^7$ leukocytes/ml peritoneal washout, (Table 3.1). Both the blood and peritoneal cavity of the immune mice contained significant numbers of eosinophils, 26% ± 5% and 18% ± 5% respectively.

The initial enrichment procedures to be described, were designed to purify eosinophils only. For this reason, peritoneal washouts were chosen as the source for eosinophils, in order to minimise neutrophil contamination, since it was thought that neutrophils may have similar characteristics to the eosinophil and therefore band at a similar density making separation of the two cell types difficult.

3.3   ENRICHMENT OF EOSINOPHILS FROM PERITONEAL WASHOUTS OF N. dubius RESISTANT MICE

A number of experiments, based on density gradient centrifugation procedures, were designed to try and separate eosinophils from the other cell types present in peritoneal washouts of mice collected 10 days after a second intravenous infection of 200 L3 given 14 days after the primary infection. Isotonic step gradients of colloidal silica: Percoll (Pharmacia) were generated by diluting 70 ml of Percoll solution with 10 ml of 0.1 M Phosphate buffered saline and 20 ml of distilled water

TABLE 3.1

Total and differential leukocyte counts on peritoneal washouts and peripheral blood from normal mice, and N. dubius infected mice, 10 days after a second immunising infection.

LACA Mice	Total number of leukocytes	Differential leukocyte counts (% $\pm$ SD.)			
		Monocytes	Lymphocytes	Neutrophils	Eosinophils
Normal					
Peritoneal cells	$2.9 \times 10^6 \pm 1.1$	97 ± 10	2 ± 1		
Blood	$5 \times 10^6 \pm 0.6$	5 ± 3	76 ± 7	19 ± 8	
N. dubius Infected					
Peritoneal cells	$12 \times 10^6 \pm 5$	70 ± 6	3 ± 2	9 ± 4	18 ± 5
Blood	$7.6 \times 10^6 \pm 0.5$	6 ± 3	15 ± 6	53 ± 10	26 ± 5

to make up a stock solution of 70% Percoll. Phosphate buffered saline 0.01M, was then added to aliquots of the 70% stock solution to generate solutions of varying concentrations of Percoll. Samples of 1 ml of four different concentrations of Percoll were then layered on top of each other, starting with the highest density, into 8 ml siliconised test tubes. Numerous gradients with varying composition were generated for example :-

(1).   35% - 40% -45% - 50% Percoll

(2).   45% - 47% -49% - 51% Percoll

(3).   45% - 50% -55% - 60% Percoll

(4).   55% - 60% -65% - 70% Percoll

Peritoneal washouts, containing $2 - 4 \times 10^7$ cells/2ml of Hanks Balanced Salt Solution were then layered onto the gradients and the cells centrifuged for 35 minutes, at 500 $\underline{g}$. Cells were collected from the interfaces of the gradients, with a pasteur pipette, placed into individual tubes and washed twice with Hanks Balanced Salt Solution containing 10% FCS. Total leukocytes in each cell fraction were counted along with the total number of eosinophils, (Table 3.2).

All attempts to separate eosinophils from the other cell types present in the peritoneal washouts of infected mice were unsuccessful. Similarly density gradients of 1 ml aliquots of Ficoll ranging from 2.7% - 5.5% were equally unsuccessful. The macrophage population within the peritoneal cavity appeared to be heterogeneous in size and density, such that these cells contaminated every cell fraction in all the gradient procedures used. Prior incubation of peritoneal cells in plastic tissue culture flasks to remove adherent macrophages prior to density gradient centrifugation failed to enrich for eosinophils.

TABLE 3.2

Enrichment of eosinophils from peritoneal washouts of N. dubius

infected mice by density gradient centrifugation procedures.

Enrichment Procedure	% eosinophils prior to enrichment	% eosinophils within the fraction giving maximum enrichment	Total cells recovered (%)
Density gradient on Percoll 35 minutes 500g			
35%-50% Percoll	20% ± 4%	11% ± 3%	ND*
45%-51% Percoll	24% ± 5%	33% ± 4%	83%
45%-60% Percoll	20% ± 4	11% ± 3%	ND*
55%-70% Percoll	20% - 4%	24% - 7%	77%
Density gradient on 2.7%-5.5% Ficoll, 50 minutes 124g	19% ± 2%	33% ± 5% some enrichment of eosinophils	94%

* ND = not done

3.4   ENRICHMENT OF POLYMORPHONUCLEAR CELLS FROM THE PERIPHERAL BLOOD OF N. dubius RESISTANT MICE

The heterogencity of the macrophage cell population, made the purification of eosinophils from the peritoneal washouts extremely difficult. For this reason, it was decided to use peripheral blood as a starting source for the enrichment of eosinophils. Monocyte levels in the blood were low, 6% ± 3%, making macrophage contamination less of a problem. A variation of the procedure described by Boyum (1968), and later by Ferrante and Thong, (1978), for the purification of human peripheral blood polymorphonuclear cells was used.

Male LACA mice received two doses of 200 L3 iv., one on day O, and the other on day 14. Mice were pre-bled via the retro-orbital plexus on day 21 (0.5 ml/mouse) and the blood discarded. This pre-bleeding ensured a cleaner separation of eosinophils after centrifugation. Peripheral blood was again collected on day 24 into lithium and heparin coated tubes. A number of different densities of Ficoll 400 containing Angiograffin and Urovison were intially tested, and the following gradient proved to be the most successful. A density gradient of three steps was prepared with a combination of Ficoll 400, Angiograffin, Urovison and Sepalymph. The density gradient was composed of :-

Part A :-   a mixture of density 1.114 g/ml was established using 10 ml of Angiograffin, 10 ml of Urovison and 58.6 ml of 10% Ficoll 400 in water.

Part B :-   Part A (70 ml) was diluted with 7.3% Ficoll, until a density of 1.096 g/ml was obtained, as measured on a hydrometer.

Part C :-   Sepalymph, density 1.076 g/ml.

Firstly, 1.3 ml of part A, then 0.9 ml of part B, and finally 1.0 ml of part C were layered gently into an 8 ml siliconised

TABLE 3.3

Total and differential leukocyte counts of the cell types present in the bands isolated from the gradient after centrifugation

Bands	Differential counts (% $\pm$ SD.)			
	Monocytes	Lymphocytes	Neutrophils	Eosinophils
1	29 ± 7	62 ± 8	1 ± 1	8 ± 4
2	8 ± 5	23 ± 7	9 ± 3	60 ± 5
3	2 ± 1	5 ± 5	89 ± 8	4 ± 4

	Leukocyte counts ($\pm$ SD.)	
	Total number of leukocytes	Total number of eosinophils
(1)	$5.3 \times 10^6 \pm 3$	$4.2 \times 10^5 \pm 4$
(2)	$4.2 \times 10^6 \pm 2$	$2.5 \times 10^6 \pm 1$
(3)	$1.6 \times 10^6 \pm 1$	$1.1 \times 10^5 \pm 1$

Results are expressed as a mean $\pm$ standard deviation of 6 experiments

FIGURE 3.1

The enrichment of murine peripheral blood eosinophils
on a density gradient.

Band 1 :   mononuclear cells

Band 2 :   predominantly eosinophils

Band 3 :   predominantly neutrophils
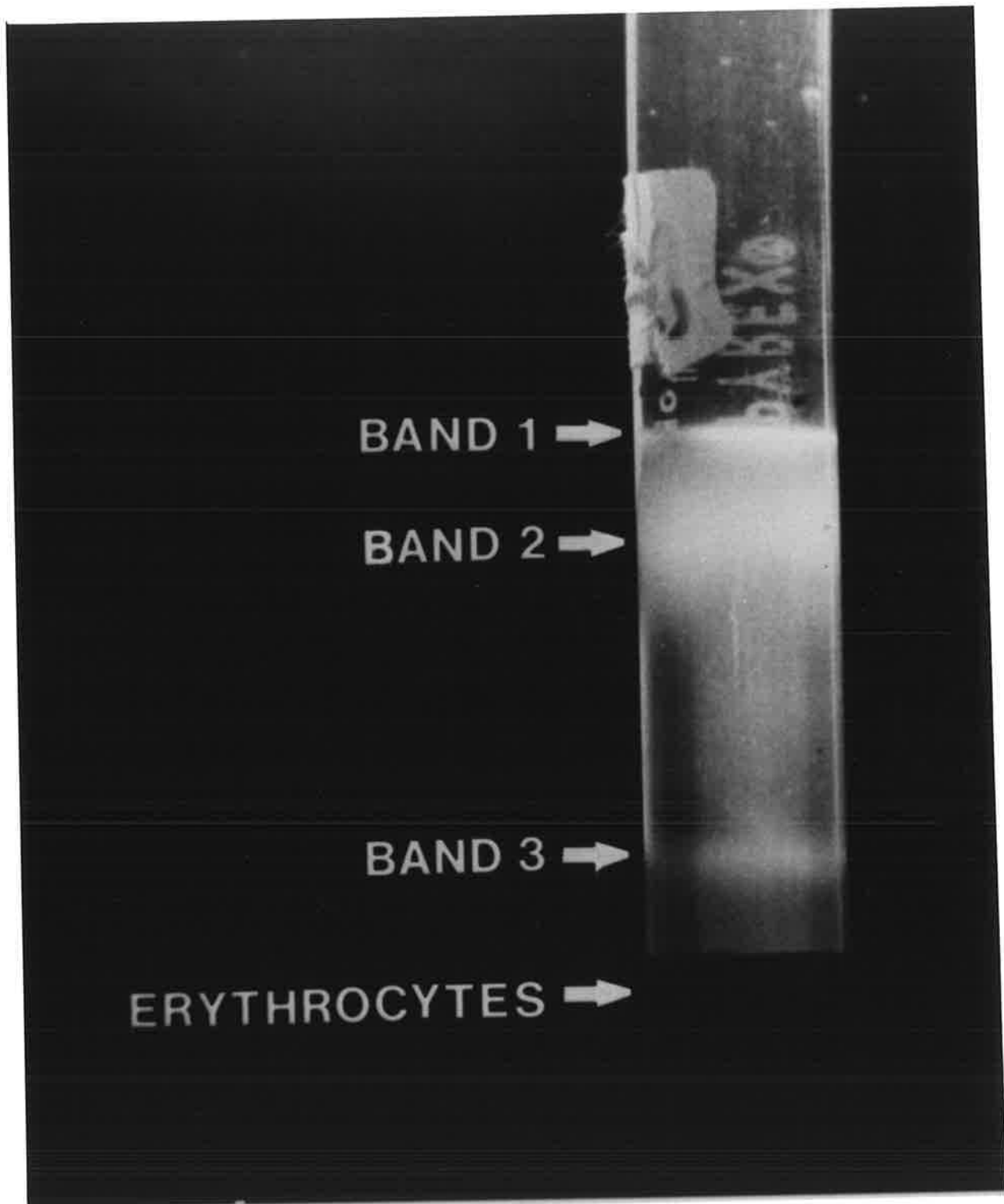
BAND 1 ➡

BAND 2 ➡

BAND 3 ➡

ERYTHROCYTES ➡

glass tubes. Pooled peripheral blood (3ml) was diluted to 5 ml in Hanks Balanced Salt Solution, layered gently onto the gradient and centrifuged at 500 g. for 40-45 minutes at room temperature. The resulting 3 bands on the gradient were removed separately with different Pasteur pipettes, and washed twice with medium 199. Differential leukocyte counts were performed on cytocentrifuge samples stained with leishman's stain. Cell viability was determined by trypan blue exclusion. Cell recovery was determined by counting in a haemocytometer before and after centrifugation.

The data in Table 3.3 and Fig. 3.1 shows that three distinct bands were obtained at the gradient interfaces after centrifugation. The top band (band 1) contained predominately mononuclear cells, the middle band (band 2) contained mononuclear cells and eosinophils, and the bottom band (band 3) contained neutrophils with only a very small percentage of mononuclear cells. Red cells went through the gradient to the bottom of the tube.

The number of leukocytes loaded onto the gradient ranged from 1.08×10^7 - 2.2×10^7 cells. After centrifugation 69% $\pm$ 11% of the total leukocytes, and more than 70% of the eosinophils were recovered in the bands. Cell viability in all cases exceeded 95%.

The gradient resulted in a cell population containing 60% $\pm$ 5% eosinophils. In addition neutrophils were also enriched by the procedure, such that a cell population containing 89% $\pm$ 8% neutrophils could be routinely obtained (Fig. 3.2).

3.5   CONCLUSIONS

Procedures to enrich eosinophils from the peritoneal washouts of N. dubius infected mice all proved to be unsuccessful, due to large numbers of contaminating macrophages. However, density centrifugation on Ficoll allowed separation of peripheral blood

FIGURE 3.2

Cyto centrifuge preparations of cells taken from (A)
Band 1 :   (B) Band 2 :   (C) Band 3 after staining with
Leishman's stain.

L   =   Lymphocyte

M   =   Monocyte

E   =   Eosinophil

N   =   Neutrophil
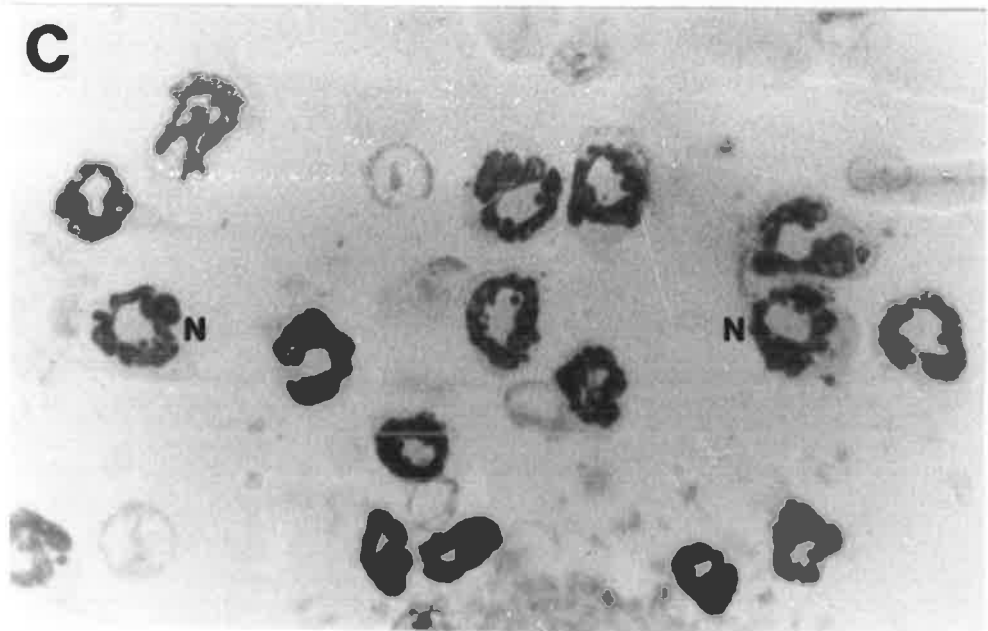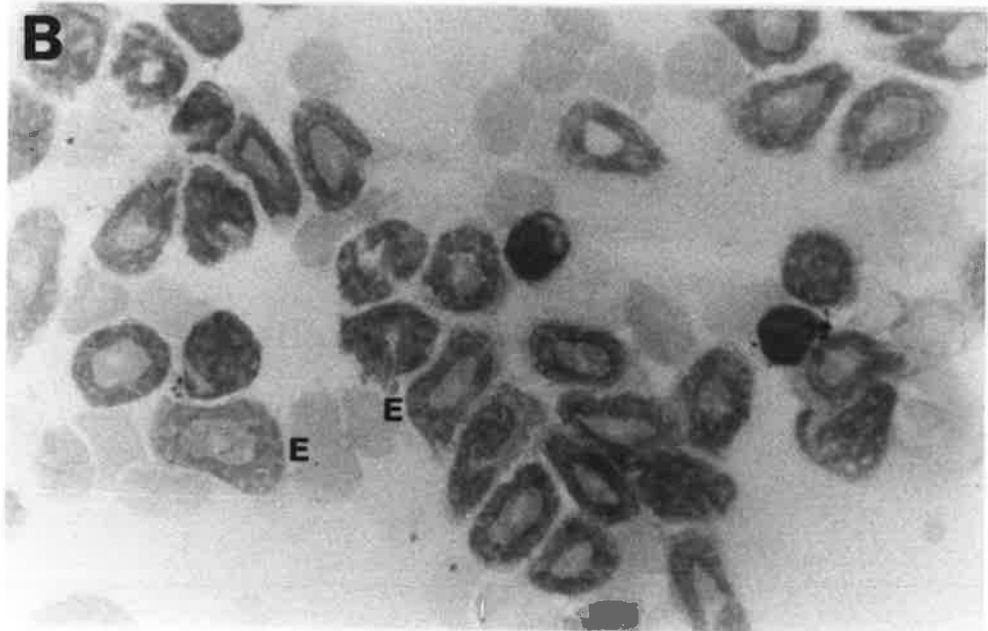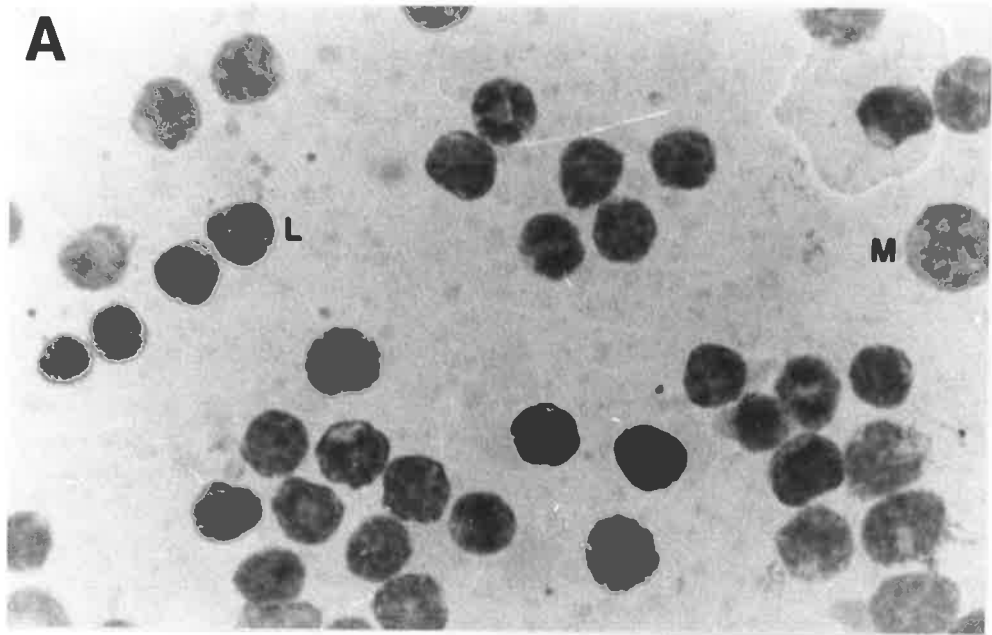
leukocytes from infected mice into fractions enriched for various cell types. Enrichment of both eosinophils and neutrophils occurred, although some neutrophils were lost into the red cell fraction before sufficient separation of the eosinophils occurred. A larger proportion of neutrophils without any contaminating eosinophils could be obtained by shortening the centrifugation time to approximately 30 minutes. The simplicity of the procedure, resulted in minimal cell loss and damage, enabling functional studies of the cells to be undertaken.

The significance of pre-bleeding the mice two days prior to collection of blood was not clear, however pre-bleeding resulted in a clearer separation of the eosinophils (Band 2) and the mononuclear cell layer (Band 1). This may be due to a release after bleeding of a more homogeneous eosinophil population into the circulation.

CHAPTER 4.

ADHERENCE OF MURINE PERIPHERAL BLOOD EOSINOPHILS AND NEUTROPHILS TO N. dubius LARVAE: ASSESSMENT OF THE ROLE OF ANTIBODY AND COMPLEMENT

One approach to the role of various cell types in immunity
to helminth infections has been to study in-vitro the adherence
of different cells to the parasite surface, the effect this
interaction has on the viability of the parasite, and the factors
that promote this adherence. It has been shown in mice immunised
against N. dubius, as a result of previous exposure to the
parasite, that a challenge dose of larvae is killed following
penetration into the muscularis mucosa of the small intestine
(Chaicumpa et al., 1977). Studies, both in-vitro and in-vivo
have implicated activated macrophages as an effector cell in
this immunity (Chaicumpa et al., 1977; Chaicumpa and Jenkin,
1978). Experiments in which millipore chambers were implanted
into the peritoneal cavity of resistant mice, demonstrated that
only direct cell contact with the parasite cuticle resulted in
a decrease in larval infectivity. The predominant cell assoc-
iated with the larval cuticle in these experiments was shown to
be the macrophage. Unfortunately, the role of other cell types
in resistance has been largely ignored even though an increase
in the number of circulating neutrophils and lymphocytes as well
as macrophages occurred after a primary infection of N. dubius.
Following a secondary infection, there was a further increase
in these cells, along with a sudden appearance of eosinophils in
the circulation, suggesting an involvement of other cell types
apart from the marcophage in immunity to N. dubius. The follow-
ing experiments were carried out to determine the relative role
of antibody and complement in promoting the adherence of
polymorphonuclear cells to the surface of the different stages in
the life cycle of N. dubius, and in particular to determine if
the factors promoting adherence of these cells differed, depend-
ing on whether the cells were obtained from normal mice, or mice
immune to reinfection with this parasite.

4.2 IDENTIFICATION OF THE ADHERENT CELL TYPES FROM THE ENRICHED EOSINOPHILS POPULATION ADHERING TO N. dubius LARVAE

Polymorphonuclear cells were purified by density gradient centrifugation from the peripheral blood of N. dubius resistant mice, 10 days after a secondary immunising infection of 200 L3 given intra-venously, 14 days after the primary infection.

The gradient resulted in three bands. Differential cell counts on the bands showed that the top band contained predominately mononuclear cells. The middle band (Band 2) contained 63% ± 3% eosinophils, 30% ± 6% lymphocytes, and 7% ± 3% monocytes (mean ± one standard deviation), whilst the bottom band (Band 3) consisted of 97% ± 2% neutrophils, 1% ± 1% eosinophils and 2% ± 1% lymphocytes. The immune serum used in all the experiments outlined in this chapter was collected from mice, which had received four immunising infections of 200 L3 intra-venously at 14 day intervals. Mice were bled 10 days after the last immunisation.

The eosinophil enriched cell population, unlike the neutrophill population, contained a significant proportion of contaminating leukocytes. Therefore, a differential count was performed on the cells from the eosinophil enriched population, adhering to the parasite surface to ensure that the greater proportion of cells adhering were eosinophils. To two tubes containing either 500 sheathed L3, or 500 exsheathed L3 in VB^{2+}, were added 0.05 ml of N. dubius immune serum. Tubes were incubated for 90 minutes at 37°C, after which time the larvae were washed twice with 5 ml of supplemented medium, and incubated with 10^6 cells, from the enriched eosinophil population in 0.2 ml of supplemented medium for 60 minutes. After incubation a drop of the suspension containing larvae and cells were placed onto a BSA coated slide, and allowed to partially dry. An equal volume

of Leishman's stain was then placed onto the drop of larvae and left for 1 minute. Finally a cover slip was placed on the slide. The total number of cells adhering to each of at least 30 larvae per tube, as well as a differential count of cell types adhering per larvae was carried out under light microscopy (1000x magnification). Of the total number of cells adhering to the parasite surface, the majority (greater than 90%) were eosinophils, with only a minor proportion of lymphoctyes (2 - 8%), or monocytes (1 - 2%) (Table 4.1 and Fig. 4.1). Figure 4.2 indicates that neutrophils from Band 3 were predominant cell type to adhere to the surface of L3.

4.3 ADHERENCE OF EOSINOPHILS TO THE LARVAL AND ADULT STAGES OF N. dubius.

To study the factors promoting the adherence of eosinophils to the surface of the larval and adult stages of N. dubius, (collected as outlined in Materials and Methods, Sections 2.4 and 2.5) were first sensitised under various conditions as out-lined below with freshly collected serum from either normal mice, or mice which had received four immunising infections of 200 L3 intra-venously. To investigate the relative importance of complement and antibody in promoting adherence of eosinophils, the medium used in this experiment contained both Ca^{2+} and Mg^{2+} ions (allowing activation of complement by the classical or alternative pathway). EGTA (permitting alternative pathway activation only), or EDTA (preventing activation of both pathways).

To six groups of 6 tubes, were added 500 worms of a particular stage in the life cycle of N. dubius. Tubes in groups 1 to 6 received either sheathed L3, exsheathed L3, 48 hour, 72 hour, 96 hour post-infective larvae, or adult worms respectively. The worms were suspended in either 0.1 ml of VB^{2+}, or VB^{2+}-EDTA or VB^{2+}-EGTA, (two tubes per group for each diluent). To two tubes in each of the groups containing worms suspended in VB^{2+}

TABLE 4.1

Analysis of the different types of cells from the eosinophil -
enriched cell population which adhere to N. dubius L3 sensitised
in VB^{2+} with immune mouse serum.

| Type of cell | Number of adherent cells* binding to | |
	Sheathed L3	Exsheathed L3
Total leukocytes	65 ± 11	99 ± 6
Monocytes	1 ± 1	1 ± 1
Lymphocytes	5 ± 2	2 ± 0
Eosinophils	59 ± 10 (91%)[+]	96 ± 6 (96%)[+]

* mean number of cells/ larva   ($\pm$ SD), determined by
differential cell counts. The cells added to the larvae comprised
eosinophils (63% $\pm$ 3%), lymphocytes (30% $\pm$ 6%) and
monocytes (7 $\pm$ 3%).

+ as a percentage of the total number of adherent cells.

FIGURE 4.1

Eosinophils adhering to the surface of
exsheathed L3 larvae.

(A) 100x magnification

(B) 400x magnification

(C) 1000x magnifciation
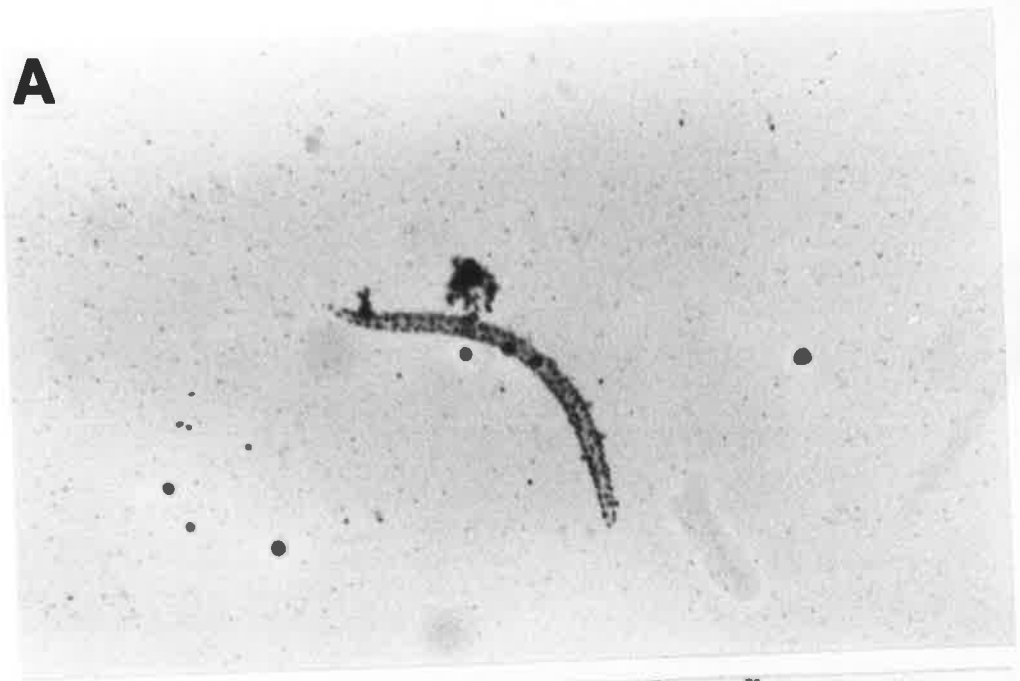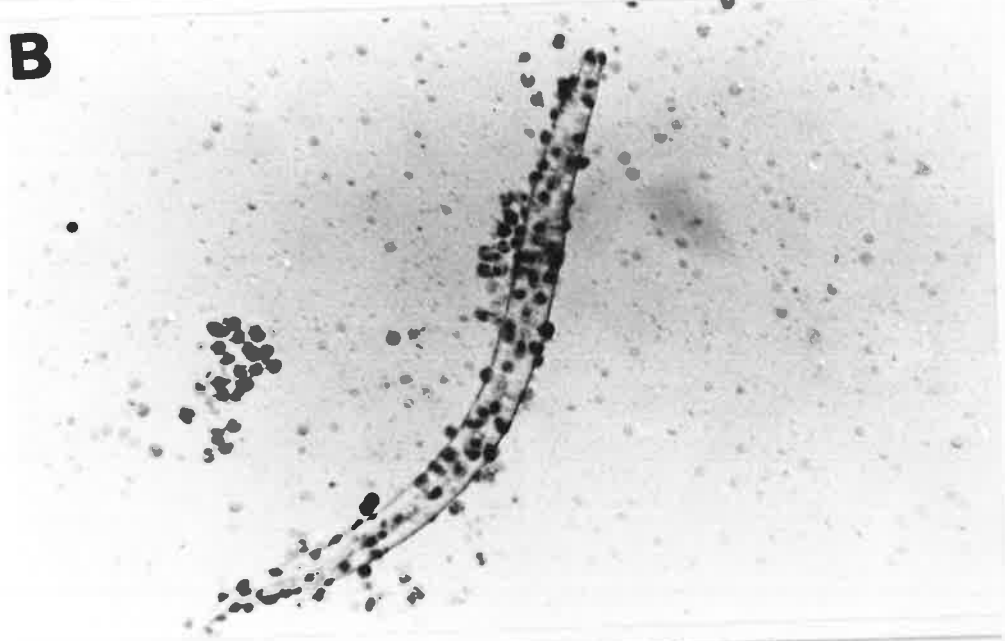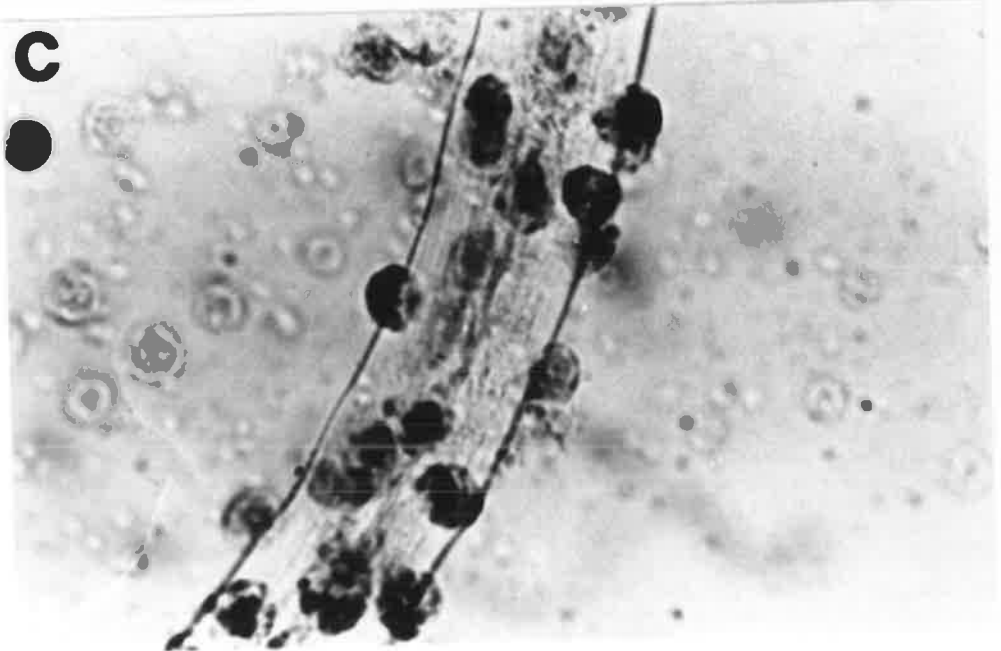
FIGURE 4.2

Neutrophils adhering to the surface of
exsheathed L3 larvae.

(A) 100x magnification

(B) 400x magnification

(C) 1000x magnification
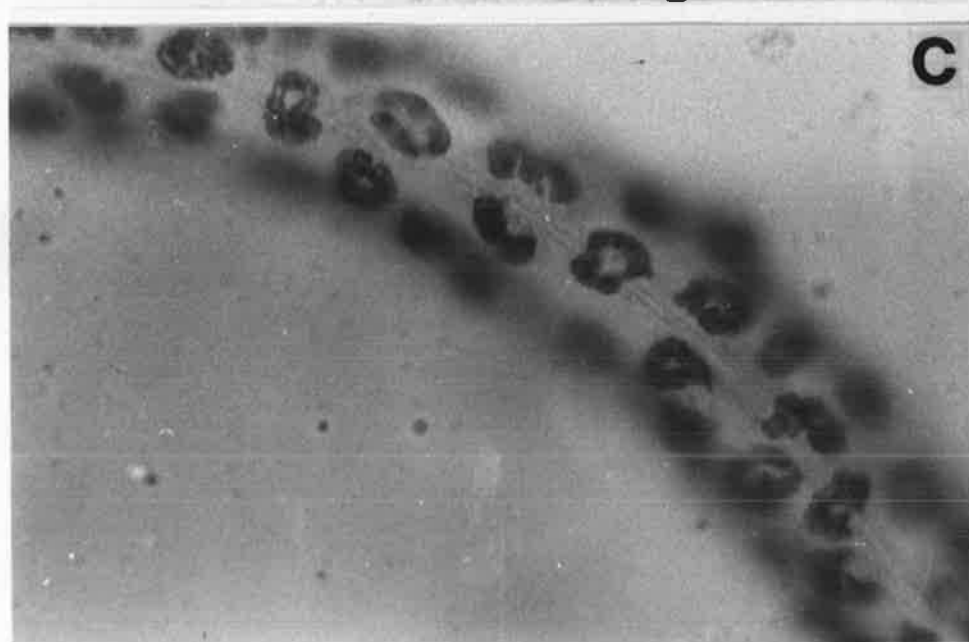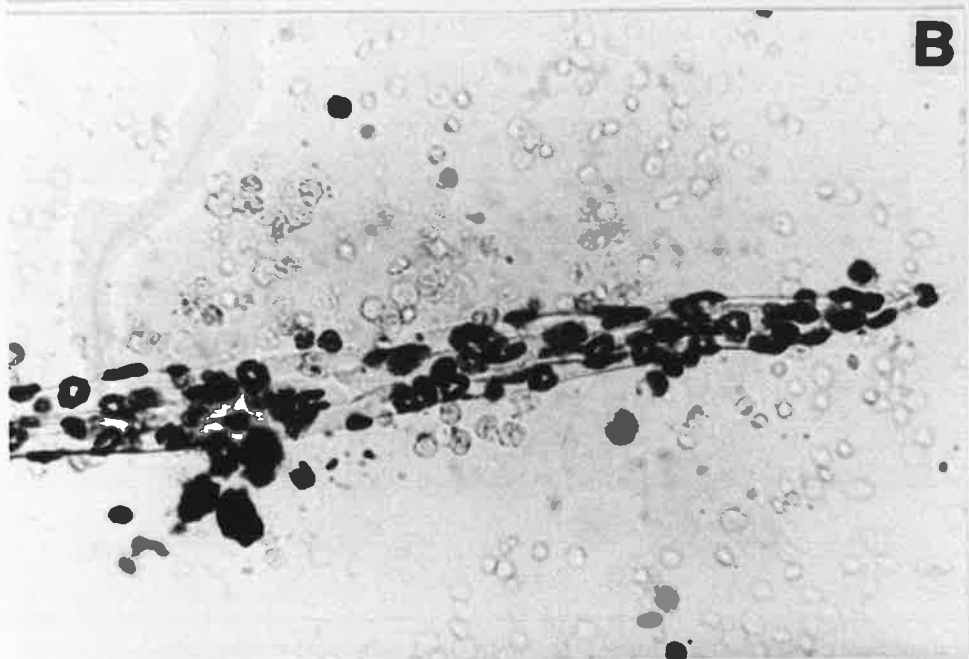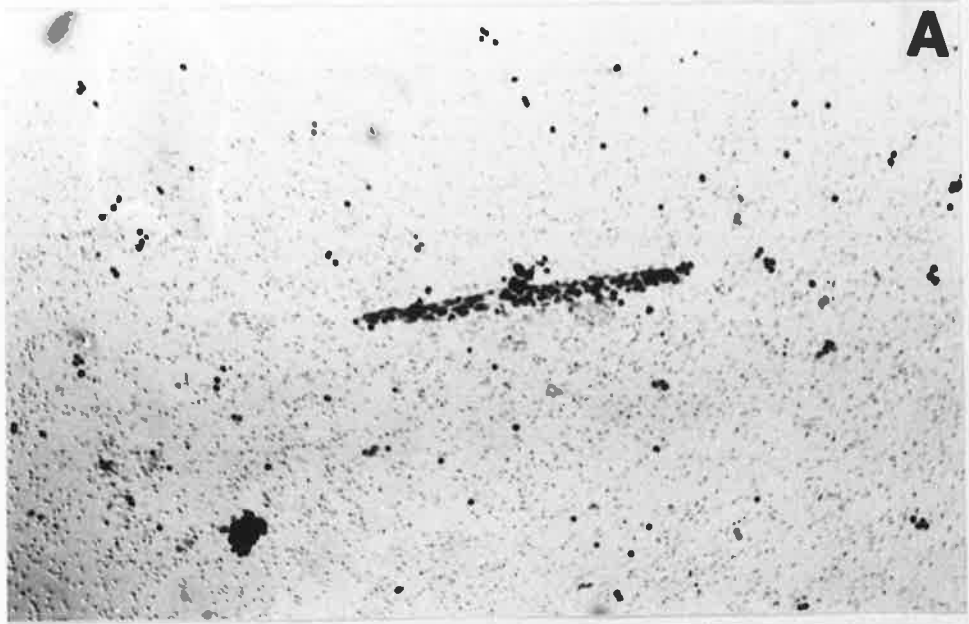
were added 0.5 ml of either immune serum or normal serum. To
a further two tubes per group containing worms suspended in
VB^{2+}-EDTA, were also added 0.5 ml of either immune or normal
serum. Finally, 0.5 ml of immune or normal serum were added to
tubes in each group containing worms suspended in VB^{2+}-EGTA.
Control tubes contained worms suspended in VB^{2+} in the absence
of serum (Table 4.2). The contents of the tubes were mixed and
incubated at 37°C for 90 minutes with gentle agitation to allow
antibodies to bind and/or complement activation to occur at the
parasite surface. After this period of incubation, each suspen-
sion of worms were washed twice with 5 ml of supplemented medium.
To the washed larvae were then added 0.25 ml of supplemented
medium containing 10^6 eosinophils, purified from the peripheral
blood of N. dubius infected mice, 10 days after a second immun-
ising infection of 200 L3. The tubes were incubated for a
further 60 minutes to allow cells to adhere to the parasite
surface. Adherence of the cells was then scored, after staining
with Leishman's stain, by counting the number of adherent cells
on 30 worms from each tube under light microscopy (1000x magnifi-
cation). The results are presented in Table 4.2.

No adherent cells could be detected on larvae that had not
been treated with serum prior to the addition of eosinophils.
However, exposure of the parasites to normal serum in the presence
of Ca^{2+}/Mg^{2+} (VB^{2+}) promoted the adherence of a large number of
eosinophils to all the stages tested. The ability of normal
serum to promote cell adherence was completely abolished by the
addition of EDTA to the serum. However, normal serum containing
EGTA was as active as normal serum in VB^{2+} in promoting adhesion,
although adherence in the presence of normal serum in EGTA-VB^{2+}
was only studied using exsheathed larvae, and not the other
parasite stages (Data not in Table). A total of 80±12 adherent
cells were counted after incubation of exsheathed L3 in normal

TABLE 4.2

Adherence of eosinophils to the larval and adult stages of

N. dubius.

Stage tested	Number of eosinophils* attached to					
	Unsensitised larvae in	Larvae sensitised with				
		Normal mouse serum in		Immune mouse serum in		
	VB^{2+}	VB^{2+}	VB^{2+}-EDTA	VB^{2+}	VB^{2+}-EGTA	VB^{2+}-EDTA
(1) Sheathed L3	0	73 ± 8	0	70 ± 6	69 ± 6	23 ± 7
(2) Exsheathed L3	0	73 ± 9	0	77 ± 8	77 ± 13	53 ± 12
(3) 48 h p.i.[+] larvae	0	93 ± 9	0	93 ± 14	87 ± 8	90 ± 14
(4) 72 h p.i. larvae	0	83 ± 13	0	92 ± 13	85 ± 10	87 ± 14
(5) 96 h p.i. larvae	0	67 ± 16	0	72 ± 13	75 ± 13	36 ± 8
(6) Adult worms	0	37 ± 9	0	40 ± 11	38 ± 12	14 ± 6

* mean number of adherent cells/ worm ($\pm$ SD), determined by total cell counts.

+ p.i. = post-infection

Figures in parenthesis represent group numbers.

serum containing VB^{2+}-EGTA, compared with 73±9 adherent cells on exsheathed L3 incubated in normal serum VB^{2+}. Since previous studies had shown that all stages in the life cycle of N. dubius were capable of activating complement by the alternative pathway, the adhesion of eosinophils to the various stages after sensitisation in normal serum VB^{2+}-EGTA was not studied (Prowse, Ey and Jenkin, 1979a). The number of cells bound to the parasites sensitised with immune serum in VB^{2+}, or in immune serum VB^{2+}-EGTA was similar to that observed with normal serum in VB^{2+}. In contrast to normal serum in VB^{2+}-EDTA where no cell adherence occurred, immune serum in VB^{2+}-EDTA promoted the binding of a large number of cells to the surface of the larval and adult stages. This was clearly due to antibodies which had bound to the surface of the parasites, since EDTA would prevent complement activation.

The adherence of eosinophils to the surface of the different larval and adult stages of N. dubius sensitised with antibody, but not complement, ie: with immune serum - EDTA showed a varied pattern. The number of adherent cells present on the surface of sheathed L3, post-infective 96 hour larvae and adult worms was great ly reduced compared with the number of cells adhering to these stages after sensitisation with immune serum - VB^{2+}, or immune serum - EGTA, indicating that complement was probably more important than antibody in promoting adhesion to these stages in-vitro. However, the titre of specific antibody immune serum against the later stages in the life cycle of N. dubius may be reduced, compared with the earlier stages. After primary immunisation of mice, larvae from subsequent immunising infections would be killed soon after penetrating the intestinal mucosa and muscularis, thus very few of the larvae would develop through to the 96 hour or adult worm stage. Mice would have only limited exposure, to these later stages and therefore may not produce a

high titre of specific antibody against these larvae. In contrast, the number of adherent cells on the surface of ex-sheathed L3 and post-infective 48 hour, and 72 hour larvae sensitised with immune serum - EDTA was similar to the number of adherent cells on the surface of these particular stages after sensitisation with immune serum - VB^{2+}. This would indicate that adhesion to these stages could be mediated as much by specific antibody, as by complement (normal serum - VB^{2+}; Table 4.2).

4.4   ADHERENCE OF NEUTROPHILS TO THE LARVAL AND ADULT
       STAGES OF N. dubius

A similar experiment to that described in section 4.3 above was repeated using neutrophils purified from the peripheral blood of N. dubius infected mice 10 days after a second immunising infection of 200 L3. As the data in Table 4.3 indicates, no differences were observed in the pattern of adherence when compared with the results obtained using eosinophils. It seems, therefore that both cell types can bind via Fc receptors and/or C3 receptors to the parasite surface.

4.5   TITRATION OF NORMAL AND IMMUNE SERUM FOR THEIR ABILITY
       TO PROMOTE ADHERENCE OF EOSINOPHILS AND NEUTROPHILS
       TO THE SURFACE OF EXSHEATHED L3

To appreciate more fully the relative role of antibody and complement in the adherence of eosinophils and neutrophils to N. dubius, a titration of the ability of normal and immune serum to promote adherence of polymorphonuclear cells to the surface of exsheathed L3 was carried out in the presence of VB^{2+}, VB^{2+}-EGTA, and VB^{2+}-EDTA.

Using the same protocol as the previous two experiments, 500 exsheathed L3 were sensitised with various dilutions of freshly collected N. dubius immune serum in the presence of VB^{2+}, VB^{2+}-EGTA, and VB^{2+}-EDTA. To six groups of 7 tubes containing

TABLE 4.3

Adherence of neutrophils to the larval and adult stages of

<u>N. dubius</u>.

	Number of neutrophils* attached to					
Stage tested	Unsensitised larvae in	Larvae sensitised with				
		Normal mouse serum in		Immune mouse serum in		
	VB^{2+}	VB^{2+}	VB^{2+}-EDTA	VB^{2+}	VB^{2+}-EGTA	VB^{2+}-EDTA
Sheathed L3	0	101 ± 10	0	96 ± 8	91 ± 4	22 ± 8
Exsheathed L3	0	91 ± 12	0	88 ± 7	85 ± 10	71 ± 4
48 h p.i.[+] larvae	0	92 ± 15	0	96 ± 10	83 ± 6	92 ± 8
72 h p.i. larvae	0	99 ± 11	0	107 ± 13	109 ± 9	81 ± 7
96 h p.i. larvae	0	62 ± 6	0	53 ± 7	44 ± 6	22 ± 6
Adult worms	0	35 ± 7	0	35 ± 8	31 ± 5	8 ± 9

* mean number of adherent cells/ worm ($\pm$SD)

+ p.i. = post-infection

FIGURE 4.3

Titration of normal and immune serum for their capacity
to sensitise larvae in the presence or absence of Ca^{2+}
and Mg^{2+} ions for the adherence of neutrophils or
eosinophils. Five hundred exsheathed L3(suspended in
0.1 ml aliquots) were incubated at 37 C with 0.1 ml
aliquots of various dilutions of normal or immune serum
before being washed and incubated with 1 x 10^6neutrophils
(o———o) or eosinophils (●———●). The incubation with
serum was carried out in VB^{2+}, plus, where indicated
10 mM EGTA or EDTA. Vertical bars represent SD.
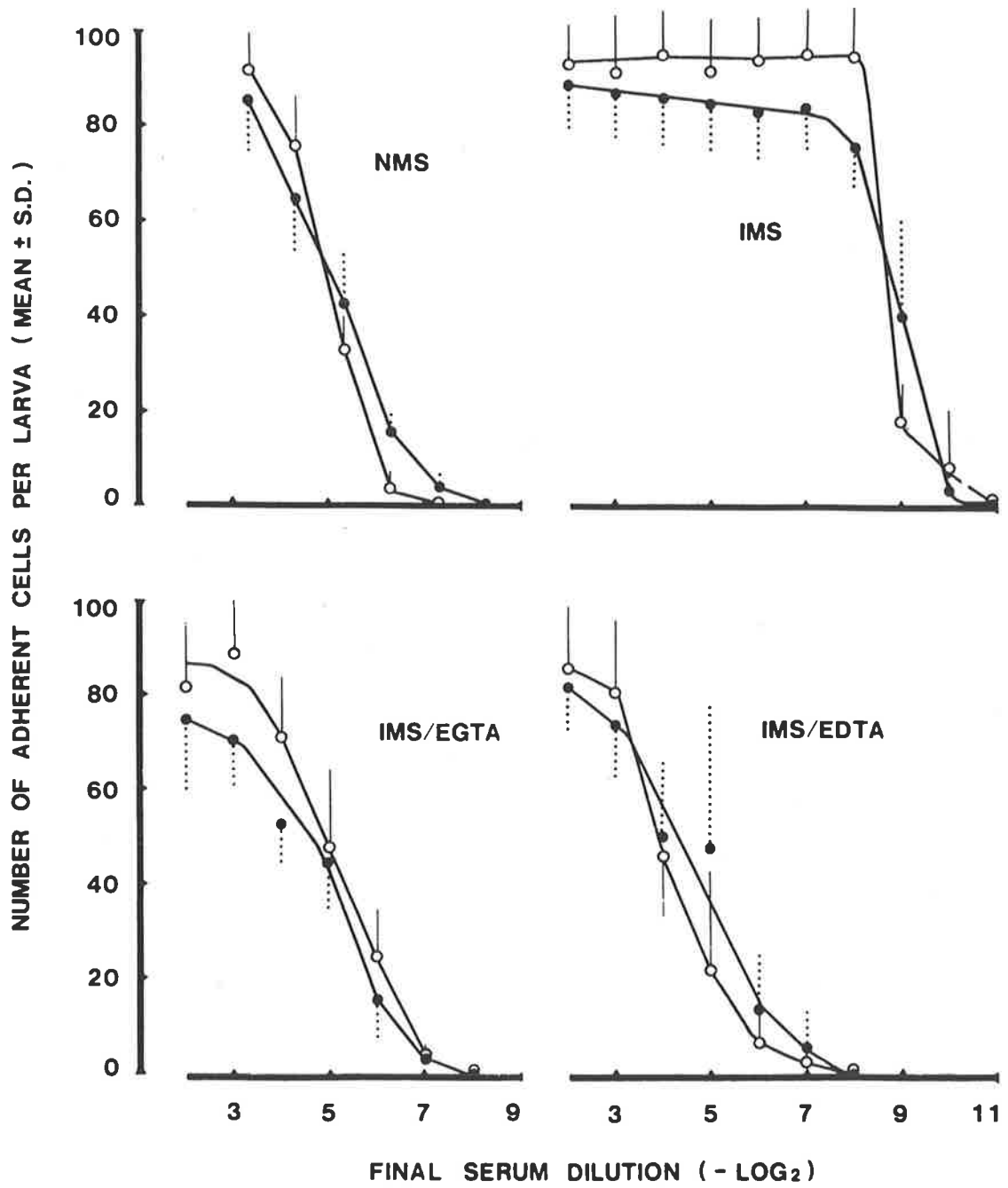
two fold dilutions (0.1 ml) of immune serum in VB^{2+} (groups
1 and 2), VB^{2+}-EGTA (groups 3 and 4) or VB^{2+}-EDTA (groups 5 and
6), were added 500 exsheathed L3 in 0.1 ml of the same buffer
that the serum dilutions were made in. To a further two groups
containing two fold dilutions (0.1 ml) of normal serum in VB^{2+}
were added 500 exsheathed L3 in VB^{2+} (groups 7 and 8). The tubes
were incubated for 90 minutes at 37°C, after which time the
larvae were washed twice in 5 ml of supplemented medium.
Neutrophils (10^6) or eosinophils (10^6), purified from the peri-
pheral blood of N. dubius infected mice 10 days after a secondary
immunising infection of 200 L3, were added to the washed larvae
in groups 1,3,5 and 7, or groups 2,4,6 and 8 respectively. The
cells were incubated with the larvae for a further 60 minutes,
after which time 30 larvae from each tube were scored for adher-
ent cells as previously described. The results were similar with
each cell type. In all serum titrations a 50% endpoint obtained
from the graphs in Fig. 4.3, represents the serum dilution result-
ing in 50 adherent cells per larvae. It can be seen from Fig.
4.3 that a similar result was obtained for normal serum in VB^{2+}
and immune serum in VB^{2+}-EGTA. Both yielded a low titre, the
50% endpoint occurring at a serum dilution of $^1/32$, consistent
with the activation of complement by the larvae via the altern-
ative pathway, which cannot function efficiently at serum
dilutions higher than about $^1/50$, (Ey, Personal communication).
Similarly, immune serum in EDTA yielded only a low titre (50%
endpoint occurring at a serum dilution of $^1/16$), suggesting that
antibodies specific for the parasite surface were limiting, and/
or that antibodies per se were not efficient in mediating cell
adhesion. In contrast, immune serum in VB^{2+} was highly efficient,
promoting the attachment of a large number of cells at a much
higher dilution (50% endpoint was at a serum dilution of $^1/400$).
This result, compared to that with immune serum - EDTA, and

immune serum - EGTA, clearly demonstrated that the antibodies which bound to the parasite were able to fix complement by the classical pathway, and thereby promote the adherence of cells via C3 receptors. This conclusion is based on the findings that Ca^{2+} was required (the effect was not seen in EGTA) and that the activity titrated out to about [1]/400, both of which are consistent with the involvement of the classical pathway.

4.6   THE ABILITY OF AN IgG AND IgM IMMUNOGLOBULIN
        FRACTION FROM IMMUNE SERUM TO PROMOTE THE
        ADHERENCE OF NEUTROPHILS AND EOSINOPHILS FROM
        N. dubius RESISTANT MICE TO THE SURFACE OF THE
        LARVAL STAGES OF THIS PARASITE

The experiments described in the previous sections demonstrated that antibodies could bind to the cuticle of N. dubius, and in addition, these antibodies promoted the adherence of eosinophils and neutrophils both via Fc/Fc-receptor interaction or by complement fixation and C3/C3-receptor interaction. The class or classes of immunoglobulin involved in promoting adherence of eosinophils and neutrophils were initially assessed using N. dubius larvae sensitised with either an IgM or an IgG fraction from N. dubius immune serum.

Worms from the different stages in the life cycle of N. dubius were sensitised with either an IgM or an IgG fraction of immune serum, obtained from serum collected 14 days after a fourth immunising infection of N. dubius as outlined in (Materials and Methods, section 2.14).

To eight groups of 6 tubes were added 500 N. dubius larvae of a given stage, in 0.1 ml of VB^{2+} - EDTA. Groups 1 and 2 contained exsheathed L3, groups 3 and 4 contained 48 hour post-infective larvae, groups 5 and 6 contained 72 hour post-infective larvae, while tubes in groups 7 and 8 contained 96 hour post-infective larvae. Furthermore, to tubes in groups 1,3,5 and 7

were added two fold dilutions of an IgG fraction of N. dubius

immune serum (0.1 ml of) VB^{2+}-EDTA, while tubes in groups 2,4,

6 and 8 received two fold dilutions of an IgM fraction of

immune serum. After fractionation of immune serum, the IgG and

IgM immunoglobulin fractions, were concentrated to a level of

15 mg/ml of IgG or 0.8 mg/ml IgM, as measured by ELISA assay.

These levels of immunoglobulin were used as the starting dilution

in the assay and were equivalent to that found in immune serum

prior to fractionation. The tubes were incubated for 90 minutes

at 37°C after which time, the larvae were washed in 5 ml of

supplemented medium. Neutrophils (10^6) purified from the blood

of N. dubius resistant mice were added to the washed larvae.

Cells were incubated with larvae for a further 60 minutes, after

which time, 30 larvae from each tube were scored for adherent

cells as previously described.

The experiment was then repeated using 10^6 eosinophils

instead of neutrophils, to assess the ability of IgG and IgM to

promote eosinophil adherence to the different larval stages.

The data in Fig. 4.4 indicates, that both eosinophils and neutro-

phils adhered in similar numbers to the larval stages of the life

cycle sensitised with the IgG fraction of immune serum. In

contrast, the IgM fraction of immune serum failed to promote the

adhesion of eosinophils and neutrophils to the cuticle of any of

the larval forms.

Adherence of eosinophils and neutrophils to exsheathed L3

via the IgG fraction of immune serum, yielded a low titre of

adherence (^{1}/4 50% endpoint). The 50% endpoint in this experiment

represents the serum dilution in the graphs in Fig. 4.4 which

resulted in half the maximum number of adherent cells per larva,

(the maximum number of cells was obtained at a serum dilution of

^{1}/2 in all cases). Exsheathed L3 in general, when sensitised with

the above immunoglobulin fraction obtained from immune serum had

FIGURE 4.4

The ability of the IgG fraction from N. dubius immune serum to promote the adherence of neutrophils (x——x) and eosinophils (●——●) to the surface of exsheathed L3, 48 hour, 72 hour and 96 hour post-infective larvae. Whole immune serum equivalent concentrations of IgG (15 mg/ml) was used in the assay. An equivalent serum concentration of IgM failed to promote adherence of cells.
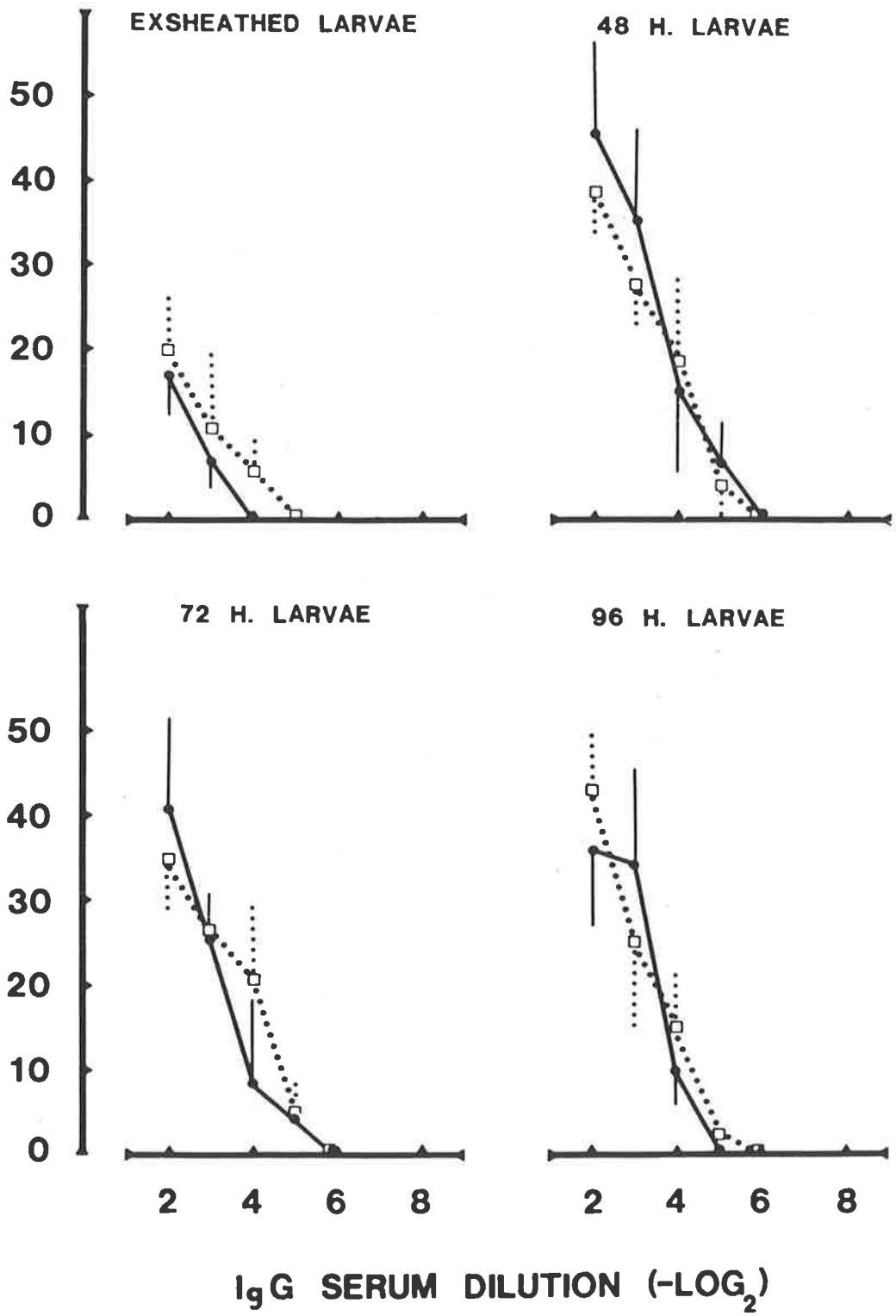
a lower number of adherent cells on their surface, when compared to similar larvae sensitised with whole immune serum in VB^{2+}-EDTA, where binding is mediated by antibody alone. One explanation for the decrease in the number of cells binding in the presence of the IgG fraction, may be due to possible denaturation of specific antibody following fractionation. The number of cells adhering to the surface of 48 hour, 72 hour, or the 96 hour post-infective larvae was twice that seen to adhere to the surface of exsheathed L3, at a ½ dilution of IgG. The adherence titre of IgG for these three post-infective larval stages, had a 50% end-point at a dilution of 1/8. This increase in titre, may indicate an increase in the antigenic determinants against which specific antibodies in the IgG immunoglobulin fraction were directed, or alternatively for these post-infective stages, a change in the antibody class mediating cell adherence may occur ie: a preferential binding of IgG over IgM. Since both eosinophils and neutrophils apparently lack receptors for the Fc portion of the IgM molecule, a preponderance of specific antibody in the IgM class would act as a blocking antibody. Alternatively one might argue that the IgM fraction lacked parasite specific antibody. However, this appears not to be the case, since IgM from N. dubius immune serum has been shown to mediate adherence of macrophages to the early stages in the life cycle of N. dubius (Desakorn, 1983).

The predominant immunoglobulin class within the IgG fraction concentrated down from immune serum was IgG_1 (15 mg/ml), with very little IgG_{2a} (1.5 mg/ml) or IgG_{2b} (0.4 mg/ml) present as measured by an ELISA assay. This corresponded to the levels of these immunoglobulins in whole unfractionated immune serum. The adherence observed in Fig. 4.4 may then be assumed to be mediated via IgG_1.

The IgG pool from immune serum was further fractionated on

a Protein A Sepharose column by the method of Ey, Prowse and Jenkin, (1978) (See Chapter 2), into an IgG_{2a} and IgG_{2b} immunoglobulin fraction, which were then concentrated to a level of 1.5 mg/ml and 0.4 mg/ml respectively. These levels were equivalent to that found in unfractionated immune serum, however at this concentration the immunoglobulins were unable to promote the adherence of eosinophils or neutrophils to the surface of the parasites. This further implicated IgG_1 as the major immunoglobulin class in promoting adherence.

4.7 THE Fc RECEPTOR SPECIFICITY OF EOSINOPHILS AND NEUTROPHILS FROM N. dubius RESISTANT MICE

The inability of neutrophils, and also eosinophils to adhere to the surface of N. dubius in the presence of immunoglobulin classes, other than IgG_1 may reflect either an absence of parasite specific antibody in these immunoglobulin classes, or alternatively the Fc receptor(s) of eosinophils and neutrophils from N. dubius resistant mice may bind IgG1 in preference to the other immunoglobulin classes. However, Lopez et al., (1981), demonstrated by using sheep erythrocytes coated with monoclonal anti-erythrocyte antibodies of various different isotypes, that murine polymorphonuclear cells ingested more erythrocytes sensitised with IgG_{2b} than they did when erythrocytes were sensitised with IgG_1, making the latter suggestion unlikely. In their studies, erythrocytes were coated with different dilutions of monoclonal antibodies of different isotypes and rosette formation, and phagocytosis assessed. However, as pointed out by the authors, it still remains uncertain whether the observed differences were as result of a greater amount of the IgG_{2b} monoclonal antibody, binding to the erythrocytes compared with IgG_1.

Since eosinophils and neutrophils from N. dubius resistant

mice were shown in the previous experiment to bind to the surface of N. dubius parasites, via an IgG fraction of immune serum, it was of interest to determine whether these cells from resistant mice lacked receptors for immunoglobulin classes other than IgG, to explain the lack of adherence seen in the presence of IgG_{2a} or IgG_{2b}. In the following experiment, the ability of eosinophils and neutrophils from N. dubius resistant mice to ingest antibody coated SRBC was assessed by a modification of the method of Lopez et al., (1981). Erythrocytes were sensitised with just less than one haemagglutinating dose of anti - SRBC antibody representing the isotypes IgG_1, IgG_{2a}, IgG_{2b}, and IgM. To 1.0 ml of an 0.5% suspension of SRBC was added 1.0 ml of a suitable dilution of anti - SRBC antibody of a given isotype. The mixtures were incubated for 30 minutes at room temperature after which time, the red cells were washed in basic medium, and re-suspended to a concentration of $5x10^7$ SRBC/ml. Antibody coated erythrocytes, in 0.1 ml of basic medium, were then added to 8 groups of 4 siliconised test tubes, groups 1 and 2 received SRBC sensitised with IgG_1, groups 3 and 4, those sensitised with IgG_{2b}, and groups 5 and 6 erythrocytes sensitised with IgG_{2a}, while groups 7 and 8 received those sensitised with IgM. Neutrophils, $(5x10^4)$ in 0.1 ml of basic medium were then added to the tubes in groups 1,3,5 and 7 and eosinophils $(5x10^4)$ to tubes in groups 2,4,6 and 8. Control tubes contained unsensit- ised SRBC in the presence of eosinophils or neutrophils. The tubes were gently centrifuged at 100 g for 2 minutes. One tube from each group was removed at times 10,20,30 and 40 minutes after incubation at 37°C, and the cells in each tube gently re- suspended. Cytocentrifuge preparations were made of the suspension and stained with Leishman's stain. The percentage of cells having ingested one or more red cells, were then counted under light microscopy (1000x magnification), a minimum of 200

TABLE 4.4

Percentage of eosinophils and neutrophils having ingested
antibody coated sheep red blood cells after varying times
of incubation.

Indicator cells sensitised with	Effector cells	Incubation time (minutes)			
		10	20	30	40
		% cells ingesting SRBC $\pm$ SD.			
IgG_1	neutrophils	29 ± 6	31 ± 3	36 ± 9	20 ± 6
	eosinophils	9 ± 3	4 ± 2	5 ± 2	7 ± 1
IgG_{2b}	neutrophils	53 ± 9	76 ± 8	66 ± 10	33 ± 8
	eosinophils	8 ± 2	17 ± 3	12 ± 6	6 ± 3
IgG_{2a}	neutrophils	13 ± 3	26 ± 5	31 ± 5	15 ± 3
	eosinophils	2 ± 1	7 ± 3	3 ± 1	2 ± 2
IgM	neutrophils	0	0	0	0
	eosinophils	0	0	0	0
unsensitised cells	neutrophils	0	0	0	0
	eosinophils	0	0	0	0

Results expressed as mean $\pm$ standard deviation of three
different experiments.

polymorphonuclear cells per field were scored for the number
of ingested erythrocytes Table 4.4. The results in Table 4.4
represent the mean ± one standard deviation of the count from
three separate experiments. For the time points above, the
majority of polymorphonuclear cells ingested only one erythro-
cyte, very rarely were there more than one erythrocyte per
polymorphonuclear cell.

No ingestion was observed of IgM coated or unsensitised
erthrocytes in control tubes by eosinophils or neutrophils,
during the period under study. For the other immunoglobulin
isotypes tested, 20-30 minutes incubation resulted in maximum
detectable phagocytosis, with neutrophils being far more effic-
ient at ingesting antibody coated erythrocytes than eosinophils
at all time points assayed. Erythrocytes coated with IgG_1,
IgG_{2a}, or IgG_{2b} were ingested by both cell types, indicating
the presence of Fc receptors capable of binding these immuno-
globulin isotypes. The longer incubation time of 20-30 minutes
contrasts to that reported by Lopez et al., (1981), who showed
that phagocytosis of opsonised red cells by polymorphonuclear
cells was at a maximum, after only 5 minutes.

4.8   CONCLUSIONS

Eosinophils and neutrophils, purified by density gradient
centrifugation from the blood of infected mice resistant to
re-infection, were tested for their ability to adhere to the
different parasitic larval stages of the murine nematode parasite
Nematospiroides dubius. Cells were tested for adherence to
larvae, which had been sensitised with immune serum or normal
serum in the presence of Ca^{2+} and Mg^{2+} ions, EDTA, or EGTA.
Differences were observed in the degree of cell adherence to the
different stages of the parasite. However, the adherence of the
two cell types to any given stage of the parasite was similar.

Adherence to the sheathed infective third-stage (L3) larvae, 96 hour post-infective larvae and to adult worms depended to a large degree on conditions suitable for complement activation (viz. fresh serum and the presence of Ca^{2+} and Mg^{2+} ions). Complement was activated both via the alternative pathway by the parasite itself and via the classical pathway by parasite-bound antibodies. In these conditions, cell adherence probably occurred predominantly through the interaction of leukocyte C3 receptors with parasite-bound C3. In contrast, adherence of cells to exsheathed L3 and to the 48 hour and 72 hour post-infective larval stages appeared to involve antibody/Fc receptor as well as C3/C3 receptor interaction. The data indicate that N. dubius may undergo a series of antigenic changes during its life cycle and that antibodies capable of mediating granulocyte attachment are elicited predominantly against the early tissue developmental forms of the parasite.

Finally, the antibody class mediating cell adherence in immune serum appears to be IgG_1. Eosinophils and neutrophils from resistant mice were capable of ingesting IgG_{2a} or IgG_{2b} coated erythrocytes, therefore these cells have Fc receptors for immunoglobulin classes other than IgG_1. The lack of adherence to N. dubius in the presence of IgG_{2a} or IgG_{2b} from N. dubius immune serum may reflect an absence of parasite specific antibody in these isotypes.

CHAPTER 5

REDUCED INFECTIVITY OF N. dubius L3 LARVAE AFTER
INCUBATION IN-VITRO WITH PURIFIED MURINE
EOSINOPHILS AND NEUTROPHILS

5.1   INTRODUCTION

Mice given a single infection of N. dubius develop a partial resistance to reinfection characterised by their ability to kill approximately 50% of a subsequent challenge dose of infective larvae (Prowse, Ey and Jenkin, 1978a). After a second immunising infection, mice develop an increased resistance, such that they can kill at least 90% of the larvae from a subsequent challenge infection. During the development of immunity the number of lymphocytes, monocytes and neutrophils increase in the peripheral blood. In particular, following a second infection, there is a sudden appearance of eosinophils in the circulation and the peritoneal cavity, coinciding with the time that the mice develop full resistance to reinfection. Previous data indicated that macrophages from immune mice were able to damage in-vitro third stage larvae of N. dubius, as measured by a loss of infectivity (Chaicumpa and Jenkin, 1978), however the precise role of polymorphonuclear cells in immunity is unclear.

The data in the previous Chapter indicated that eosinophils and neutrophils were capable of adhering to the surface of N. dubius larvae, via both antibody and complement dependent mechanisms. The work outlined in this Chapter assessed the ability of eosinophils and neutrophils in-vitro to impair the infectivity of exsheathed L3 under different serum conditions.

5.2   THE EFFECT OF INCUBATING NEUTROPHILS AND EOSINOPHILS FROM RESISTANT MICE FOR DIFFERENT PERIODS OF TIME WITH EXSHEATHED L3 IN THE PRESENCE OF IMMUNE SERUM ON THEIR INFECTIVITY

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

TABLE 5.1

Effect of incubating polymorphonuclear cells from N. dubius resistant mice for varying periods of time with exsheathed L3 in the presence of immune serum on their infectivity.

Serum added	Cells added	Time of incubation (hours)		
		0	24	48
		cyst counts $\pm$ SD		
None	None	(1) 164 ± 8	(2) 158 ± 5	(3) 154 ± 10
Immune serum	None		(4) 151 ± 6	(5) 149 ± 8
Immune serum	Neutrophils		(6) 138 ± 6	(7) 69 ± 10
Immune serum	eosinophils		(8) 141 ± 9	(9) 75 ± 8

Figures in parenthesis represent group numbers.

Group 4 vs. 6 and 8 $P > 0.05$ not significant

Group 5 vs. 7 and 9 $P < 0.05$

('U' test of Mann and Whitney)

Nine groups of 5 tubes were set up containing 200 exsheathed L3 in 0.2 ml of supplemented medium. To tubes in groups 4 and 9 were added 0.05 ml of immune serum. Control groups 1, 2 and 3 received 0.05 ml of medium alone. After incubation at 37°C for 90 minutes, 10^6 neutrophils in 0.2 ml supplemented medium were added to groups 6 and 7, while 10^6 eosinophils were added in a similar quantity of medium to tubes in groups 8 and 9. Control groups 4 and 5 received 0.2 ml of supplemented medium alone. The tubes in groups 2, 4, 6 and 8 were incubated for a further 24 hours, after which time *while tubes 3, 5, 7 and 9 were incubated for 48 hours* the contents were fed to mice and cysts counted 6 days later.

The data in Table 5.1 indicates there was no loss of infectivity of unsensitised larvae, or immune serum sensitised larvae. In contrast the infectivity of larvae was significantly reduced, following incubation with either eosinophils or neutrophils in the presence of immune serum over a period of 48 hours, but not 24 hours. Therefore in the following experiments 48 hours was chosen as the standard incubation time.

5.3   THE CAPACITY OF EOSINOPHILS OR NEUTROPHILS FROM
       MICE INFECTED WITH N. dubius TO IMPAIR THE
       INFECTIVITY OF EXSHEATHED L3

Neutrophils and eosinophils, isolated from the blood of mice resistant to reinfection by N. dubius were assessed for their ability to damage and impair the infectivity of exsheathed larvae in-vitro. In all the following experiments, exsheathed larvae were used. Furthermore, cells and serum were always used fresh within 3-4 hours of collection.

To determine the minimum number of cells required to damage and impair the infectivity of exsheathed larvae in the presence of immune serum, the following experiment was carried out. Seven groups of five tubes each were set up. The tubes, containing larvae (200L3) in 0.2 ml of supplemented medium

plus 0.05 ml of <u>N. dubius</u> immune serum were incubated at 37°C for 90 minutes, to allow for complement activation and also antibody binding to the parasite surface. To each tube in groups 1-4 were then added 0.2 ml of supplemented medium containing either 10^3, 10^4, 10^5 or 10^6 neutrophils which had been obtained by the method reported in Chapter 3. To groups 5-7, 10^3, 10^4, 10^5 or 10^6 eosinophils were added respectively. The suspensions were incubated for a further 60 minutes, to allow cells to adhere to the larval surface. Finally 5 ml of supplemented medium were then added, and the incubation continued for a further 48 hours. Control groups in the experiment contained exsheathed larvae either alone, or larvae plus immune serum in the absence of cells, or larvae plus 10^6 eosinophils, or 10^6 neutrophils in the absence of serum. The control tubes were incubated for the same period of time as the experimentals. Loss of infectivity of larvae was assessed after 48 hours, by feeding the contents of the tubes from the different groups to naive mice and counting encysted larvae in the intestinal wall 6 days later. The results in Table 5.2 show that neither immune serum nor cells alone, had any effect on the infectivity of the larvae. However, both neutrophils and eosinophils in the presence of immune serum damaged the larvae as measured by a loss of infectivity. As few as 10^4 neutrophils or 10^5 eosinophils added to 200 L3 were capable of damaging the larvae, although the greatest effect was achieved using 10^6 cells. Accordingly, unless otherwise stated in the text, 10^6 cells were used in all of the following experiments.

5.4   <u>SERUM FACTORS INVOLVED IN THE KILLING OF EXSHEATHED</u>
        <u>L3 BY POLYMORPHONUCLEAR CELLS</u>

The data in Chapter 4 indicated that both antibody and complement promoted the adherence of eosinophils and neutrophils

TABLE 5.2

The ability of eosinophils or neutrophils from infected mice
to impair the infectivity of exsheathed N. dubius L3 in the
presence of immune serum.

Number of cells added	Type of cell added	
	Eosinophils	Neutrophils
	mean cyst count $\pm$ SD.	
None(control)	$^\$$ 164 ± 6 (153 ± 5)*	
1×10^3	Not done	147 ± 14
1×10^4	154 ± 13	118 ± 9 #
1×10^5	109 ± 7 #	96 ± 10 #
1×10^6	99 ± 7 #	83 ± 13 #
1×10^6 (no IMS)	160 ± 5**	160 ± 5**

$ L3 incubated in medium alone

* L3 incubated in medium along with IMS

** L3 incubated with cells in the absence of IMS

P vs. control group * (153 ± 5) < 0.05 ('U' test of Mann and
Whitney)

to the surface of N. dubius larvae and adult worms. Further-
more the previous results (Section 5.2) showed that these
cells were also capable of killing exsheathed larvae in-vitro
in the presence of fresh serum from mice immune to N. dubius.
To determine whether both antibody and or complement were needed
to promote damage to the larvae by polymorphonuclear cells, the
following experiment was carried out. Ten groups of 5 tubes
containing 200 exsheathed L3 in 0.2 ml of supplemented medium
were set up. To tubes in groups 1 and 2 were added 0.05 ml of
fresh N. dubius immune serum, to tubes in groups 3 and 4, 0.05
ml of fresh normal serum, while to tubes in groups 5 and 6,
0.05 ml of heat-inactivated immune serum. Control groups 7 to
10 contained respectively, either larvae alone, or larvae in
the presence of (a) immune serum, (b) normal serum, or (c) heat-
inactivated immune serum.

The protocol for this experiment was essentially similar
to the previous one. To groups 1, 3 and 5 were added 10^6
neutrophils, whilst to groups 2, 4 and 6 were added 10^6
eosinophils. After 48 hours incubated at 37°C, the contents of
the tubes were fed to mice and the viability of the larvae
assessed from cyst counts on day 6. The data in Table 5.3 shows
that neutrophils can impair the infectivity of exsheathed larvae
in the presence of specific antibody and complement (immune
serum), or complement alone (normal serum), but not in the
presence of specific antibody alone (heat-inactivated immune
serum). Eosinophils in contrast, required the presence of both
antibody and complement for maximal effect. However, eosinophils,
like neutrophils were unable to damage and impair the infectivity
of larvae in the presence of antibody alone.

TABLE 5.3

Analysis of serum requirements for the <u>in-vitro</u> damage of exsheathed <u>N. dubius</u> L3 by eosinophils or neutrophils from infected mice.

Serum added	None	Neutrophils(10^6)	Eosinophils(10^6)
		Cells added	
		mean cyst counts $\pm$ SD	
None(control)	(7) 152 $\pm$ 10		
Immune serum	(8) 138 $\pm$ 10	(1) 75 $\pm$ 13	(2) 107 $\pm$ 13
Normal serum	(9) 149 $\pm$ 10	(3) 91 $\pm$ 8	(4) 131 $\pm$ 5
Heat-inactivated immune serum	(10) 147 $\pm$ 5	(5) 147 $\pm$ 5	(6) 147 $\pm$ 5

Figures in parenthesis represent group numbers

Group 8 vs. groups 1 and 2 P < 0.05

Group 9 vs. groups 3 and 4 P < 0.05

Group 10 vs. groups 5 and 6 P > 0.05 not significant

('U' test of Mann and Whitney)

5.5   EFFECT ON THE INFECTIVITY OF EXSHEATHED L3
BY A COMBINATION OF EOSINOPHILS AND NEUTROPHILS

Enriched populations of eosinophils and neutrophils were
capable of damaging and impairing the infectivity of larvae,
providing antibody and/or complement were present in the assay.
Histopathological studies (Jones and Rubin, 1974) have shown
both cell types to be present and associated with encysted
larvae within the intestinal muscularis mucosa of N. dubius
resistant mice. It was therefore decided to try a combination
of these two cell types in an in-vitro larvicidal assay to
determine whether an enhanced reduction in larval infectivity
could be achieved. Five groups of 5 tubes were set up containing
200 exsheathed L3 in 0.2 ml of supplemented medium. To tubes
in groups 2, 3, 4 and 5 were added 0.05 ml of fresh immune
serum. Control group 1 received 0.05 ml of medium alone. After
incubation at 37°C for 90 minutes to allow for the binding of
antibody and complement activation at the parasite surface,
10^6 eosinophils in 0.2 ml of supplemented medium were added to
tubes in group 3, while tubes in group 4 received 10^6 neutrophils.
Tubes in group 5 received a combination of 5×10^5 eosinophils,
plus 5×10^5 neutrophils in 0.2 ml of supplemented medium.
Control groups 1 and 2 contained larvae in medium alone, or
larvae plus immune serum respectively. The tubes were incubated
for a further 48 hours, after which time the contents were fed
to mice and the cysts counted in the intestinal wall 6 days
later. The data in Table 5.4 indicates that whilst enriched
populations of either neutrophils or eosinophils were capable
of damaging exsheathed larvae in the presence of immune serum
to a significant degree, no enhancement of damage occurred when
a combination of eosinophils and neutrophils were used in the
larvicidal assay.

TABLE 5.4

Larvicidal activity of neutrophils and eosinophils, or a combination of both cell types in the presence of fresh immune serum

Serum added	Cells	added		
	None	Eosinophils(10^6)	Neutrophils(10^6)	Eosinophils + Neutrophils (5×10^5 of each)
		mean cyst counts $\pm$ SD		
None	(1) 165 $\pm$ 7			
Immune serum	(2) 160 $\pm$ 3	(3) 132 $\pm$ 6	(4) 116 $\pm$ 5	(5) 121 $\pm$ 5

Figures in parenthesis represent group numbers

Group 1 vs. groups 3, 4 and 5 < P 0.05

Group 2 vs. groups 3, 4 and 5 < P 0.05

('U' test of Mann and Whitney)

5.6   EFFECT OF PERIPHERAL BLOOD LYMPHOCYTES AND MONOCYTES ON LARVAL INFECTIVITY

To eliminate the possibility that contaminating lymphocytes in the polymorphonuclear cell populations were capable of mediating damage to exsheathed larvae, an in-vitro larvicidal assay was carried out using cells from Band 1, from the density gradient described in Chapter 3. Cells were purified from the peripheral blood of N. dubius infected mice, by density centrifugation. Cells from Band 1 and also Band 3, were compared in regard to their larvicidal activity in the presence of normal and immune serum. The cell population in Band 1 consisted of 30% ± 8% monocytes, and 64% ± 9% lymphocytes. The cell population in Band 3 consisted of 97% ± 1% neutrophils, 2% ± 1% lymphocytes, and 1% ± 1% monocytes. Seven groups of 5 tubes were set up containing 200 exsheathed larvae in 0.2 ml of supplemented medium. To tubes in groups 1 - 3 were added 0.05 ml immune serum, and to those in groups 4 - 6, 0.05 ml of normal serum. After incubation at 37°C for 90 minutes, 10^6 neutrophils in 0.2 ml supplemented medium (Band 3 cells) were added to groups 3 and 6, while 10^6 lymphocytes and monocytes in 0.2 ml of supplemented medium (Band 1 cells) were added to groups 2 and 5. Control groups 1 and 4 contained larvae plus immune serum, or normal serum respectively. The tubes were incubated for a further 48 hours, after which the contents were fed to mice, and cysts counted 6 days later.

The data in Table 5.5 indicates, that lymphocytes and monocytes from peripheral blood, at a concentration far exceeding the level found to contaminate the polymorphonuclear cell populations used in the experiments described in this Chapter, were unable to impair the infectivity of N. dubius larvae in-vitro in the presence of antibody and/or complement.

TABLE 5.5

Larvicidal activity of cells derived from Band 1 and Band 3 after centrifuging peripheral blood from N. dubius infected mice on a density gradient

Serum added	None	Band 1*	Band 3*
		10^6 cells added	
		mean cyst counts $\pm$ SD	
Immune serum	(1) 161 ± 20	(2) 142 ± 12	(3) 89 ± 5
Normal serum	(4) 163 ± 12	(5) 148 ± 15	(6) 100 ± 7
None	(7) 165 ± 10	ND	ND

ND = not done

* Band 1 contained $30\% \pm 8\%$ monocytes, $64\% \pm 9\%$ lymphocytes $1\% \pm 1\%$ neutrophils, and $5\% \pm 3\%$ eosinophils

* Band 3 contained $2\% \pm 1\%$ lymphocytes, $1\% \pm 1\%$ monocytes and $97\% \pm 1\%$ neutrophils

Figures in parenthesis represent group numbers

Group 1 vs. group 3 $P < 0.05$

Group 1 vs. group 2 $P > 0.05$ not significant

Group 4 vs. group 6 $P < 0.05$

Group 4 vs. group 5 $P > 0.05$ not significant

Group 7 vs. groups 3 and 6 $P < 0.05$

('U' test of Mann and Whitney)

5.7   IMPAIRMENT OF LARVAL INFECTIVITY BY NEUTROPHILS FROM MICE EITHER INFECTED WITH N. dubius OR FROM NORMAL MICE

In view of the ability of neutrophils from immune mice, which had received two immunising infections of N. dubius to impair the infectivity of exsheathed L3, it was of interest to determine whether neutrophils with larvicidal activity arose after a primary infection, and also to determine if neutrophils from normal mice also possessed larvicidal properties.

Using the same experimental procedure as before, 13 groups of 5 tubes were set up, each containing 200 exsheathed L3 in 0.2 ml of supplemented medium. To groups 1, 4 and 7 were added 0.05 ml of normal serum, to groups 2, 5 and 8, 0.05 ml of immune serum, while to groups 3, 6 and 9, 0.05 ml of heat-inactivated immune serum. Finally after 90 minutes incubation at 37°C, 10^5 neutrophils from normal mice were added to groups 1 - 3, 10^5 neutrophils harvested from mice 10 days after primary infection of N. dubius were added to groups 4 - 6, and to groups 7 - 9 were added 10^5 neutrophils from the peripheral blood of mice 10 days after a second immunising infection of N. dubius larvae. Control groups 10 - 13 contained larvae incubated in the presence of medium alone, normal serum, immune serum, or heat-inactivated serum respectively.

Based on cyst counts on day 6, following the feeding of the contents of the tubes to naive mice (Table 5.6), it was clear that neutrophils from both groups of immunised mice, damaged the larvae when incubated together in the presence of immune serum, normal serum, but not when incubated in heat-inactivated immune serum. In contrast, neutrophils from normal mice caused no reduction in cyst numbers in the presence of immune serum or normal serum.

TABLE 5.6

Comparison of the larvicidal properties of neutrophils from uninfected and primary, or secondary N. dubius infected mice.

Serum added	Neutrophils added (10^5 per tube)			
added	None	Normal	1x infected	2x infected
		mean cyst count $\pm$ SD		
None	(10) 155 $\pm$ 9			
Normal serum	(11) 159 $\pm$ 5	(1) 154 $\pm$ 3	(4) 97 $\pm$ 8	(7) 92 $\pm$ 7
Immune serum	(12) 167 $\pm$ 3	(2) 141 $\pm$ 9	(5) 86 $\pm$ 3	(8) 82 $\pm$ 7
Heat inactivated immune serum	(13) 164 $\pm$ 4	(3) 161 $\pm$ 5	(6) 166 $\pm$ 5	(9) 164 $\pm$ 4

Figures in parenthesis represent group numbers

Group11 vs. groups 4 and 5 P = 0.008

Group 12 vs. groups 5 and 8 P = 0.008

Group 13 vs. groups 6 and 9 P>0.05 not significant

Group 11 vs. group 1 , and group 12 vs. group2 P>0.05

('U' test of Mann and Whitney)

Since it was difficult to purify neutrophils in sufficient quantities from the peripheral blood of normal mice, a lower number (10^5) cells/tube were used. It could be argued, that if one had used 10^6 neutrophils from normal mice per tube, some effect on the larvae may have been observed. Nevertheless, it is clear that as a population, neutrophils from mice infected with N. dubius have a greater larvicidal potential than those obtained from normal mice.

5.8   THE TIME OF APPEARANCE OF NEUTROPHILS ABLE TO
        IMPAIR THE INFECTIVITY OF N. dubius L3 IN MICE
        INFECTED WITH N. dubius

The following experiment was carried out to determine the time of appearance of neutrophils in the peripheral blood capable of damaging exsheathed L3. Two groups of 25 mice were given a primary infection of 200 L3, and 14 days later a secondary infection. One of these groups was to be bled for neutrophils, 2 weeks after the secondary infection, and the other after 6 weeks. A further five groups of mice were given a primary infection of 200 L3, and one group of mice bled on the day of infection, or days 2, 4, 7 and 10, after infection. The experiment was arranged, such that irrespective of the time lapse between the challenges, all groups of mice were bled on the same day. Seven groups of five tubes were prepared representing the seven groups of mice.

To each tube were added 200 exsheathed L3 in 0.2 ml of supplemented medium, 10^6 neutrophils in a similar volume and finally 0.05 ml of freshly-collected immune serum. A control series of tubes contained larvae in the presence of immune serum, but without cells. Following the experimental design previously outlined, the contents of each tube were incubated for 48 hours at 37°C and then fed to mice, which were killed 6 days later for cyst counts. The number of cysts present in

animals that had been fed larvae, which had been in contact
with neutrophils, is expressed in Figure 5.1 as a percentage
of those observed in mice fed larvae from tubes containing
supplemented medium and immune serum alone (no cells). It is
clear from the data, that neutrophils able to impair larval
infectivity were present in detectable numbers within 4 days
of a primary infection.

5.9   EFFECT OF NEUTROPHILS FROM MICE INFECTED WITH
        SALMONELLA enteritidis 11RX ON THE INFECTIVITY
        OF N. dubius L3 IN-VITRO

Neutrophils from N. dubius infected mice, compared with
those from normal mice, were capable of impairing the infectivity
of L3 in-vitro. Intra-peritoneal immunisation of mice with
S. enteritidis 11RX has been reported to result in a peritoneal
cell population, also capable of impairing the infectivity of
N. dubius L3 in-vitro (Chaicumpa and Jenkin, 1978). Infection
with S. enteritidis 11RX gives rise to a population of activated
macrophages, which were thought to be responsible for the
in-vitro larvicidal activity reported above. However, neutrophils
present within the peritoneal washouts of S. enteritidis 11RX
infected mice may also be in a 'stimulated' state and therefore
may also have contributed to the results obtained by Chaicumpa
and Jenkin, (1978). To assess whether an unrelated infection
with S. enteritidis 11RX was capable of inducing 'stimulated'
neutrophils and larvicidal activity, the following experiment
was carried out.

Neutrophils were purified from the peripheral blood of
mice 8 days after an intra-peritoneal injection of 10^5
S. enteritidis 11RX. Eight groups of 5 tubes were set up
containing 200 exsheathed L3 in 0.2 ml of supplemented medium.

FIGURE 5.1

The appearance in infected mice of 'stimulated' neutrophils
capable of impairing the infectivity of exsheathed L3.
Neutrophils were isolated from the blood collected at the
times indicated after a primary or a secondary infection
with 200 L3. The cells (10^6 per tube) were incubated in-vitro
with exsheathed L3 in the presence of immune serum. The
number of surviving larvae, measured as day 6 cyst counts,
in each group is depicted as a percentage of that observed
for larvae incubated in immune serum in the absence of cells.
Vertical bars represent $\pm$ standard deviation.
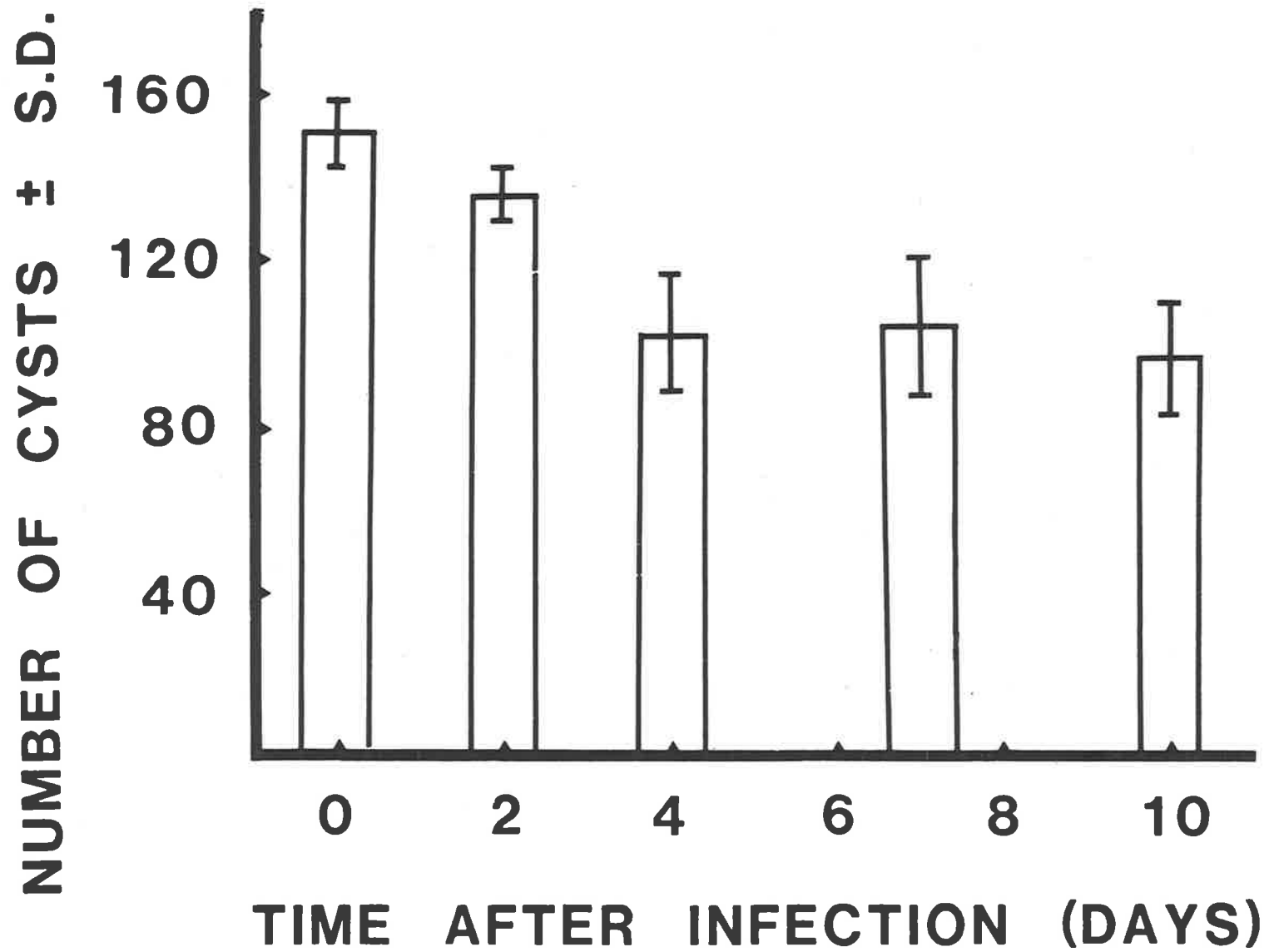
TABLE 5.7

Effect of neutrophils from mice infected with S. enteritidis 11RX
on the infectivity of N. dubius L3 in-vitro

Serum	Cells added	
added	None	Neutrophils (5×10^5)
	mean cyst counts $\pm$ SD	
None	(1) 153 $\pm$ 15	(5) 163 $\pm$ 7
Normal serum	(2) 164 $\pm$ 13	(6) 122 $\pm$ 13
* Immune serum	(3) 158 $\pm$ 6	(7) 117 $\pm$ 10
Heat-inactivated immune serum	(4) 163 $\pm$ 7	(8) 155 $\pm$ 15

Figures in parenthesis represent group numbers

Group 2 vs. 6 P<0.05 , Group 3 vs. 7 P<0.05 , Group 4 vs. 8 P>0.05

('U' test of Mann and Whitney)

* Immune serum from mice immune to N. dubius

To tubes in groups 2 and 6 were added 0.05 ml of normal serum, and to those in groups 3 and 7, 0.05 ml of normal serum, whilst to groups 4 and 8 were added 0.05 ml of heat-inactivated immune serum. After incubation at 37°C for 90 minutes, 5×10^5 neutrophils from S. enteritidis 11RX infected mice in 0.2 ml of supplemented medium were added to tubes in groups 5, 6, 7 and 8. Control groups 1, 2, 3 and 4 received 0.2 ml of medium alone. The tubes were incubated for a further 48 hours, after which the contents were fed to mice and cysts counted 6 days later.

The data in Table 5.7 indicates that immunisation of mice with S. enteritidis 11RX. which is known to activate macrophages, also resulted in the appearance of neutrophils in the peripheral blood with larvicidal activity. This activity being expressed only in the presence of antibody and/or complement. Thus the appearance of neutrophils having an increased larvicidal activity is not a unique phenomena, associated with N. dubius infection.

5.10   THE TIME OF APPEARANCE OF NEUTROPHILS ABLE TO IMPAIR
        THE INFECTIVITY OF N. dubius L3, IN MICE INFECTED
        WITH S. enteritidis 11RX

The following experiment was carried out to determine the time of appearance of neutrophils in the peripheral blood, of mice after S. enteritidis 11RX infection, capable of damaging exsheathed L3 in-vitro. The protocol was as described in Section 5.8, except mice were bled from the retro-orbital plexus at the time of challenge with 1×10^5 S. enteritidis 11RX ip., and 2, 4 and 8 days later. Neutrophils (10^6) from these mice were added to 4 groups of 5 tubes containing 200 L3 in 0.2 ml of supplemented medium, which had been incubated with 0.05 ml of immune serum from N. dubius immune mice for 90 minutes. A control series of 5 tubes contained larvae in supplemented medium alone, or in 0.05 ml of immune serum in supplemented medium in the absence

FIGURE 5.2

Time of appearance of neutrophils able to impair the infect-
ivity of N. dubius L3, in mice infected with S. enteritidis
11RX. Neutrophils were isolated from the blood of infected
mice at the times indicated. The cells were incubated in-vitro
with exsheathed L3 in the presence of N. dubius immune serum.
The number of surviving larvae, was measured as day 6 cyst
counts. P values vs. control (day 0) cyst counts.
$P < 0.05$ for days 4 and 8, $P > 0.05$ for day 2
('U' test of Mann and Whitney)

% OF CYSTS RELATIVE TO CONTROL

TIME AFTER IMMUNISATION (DAYS)
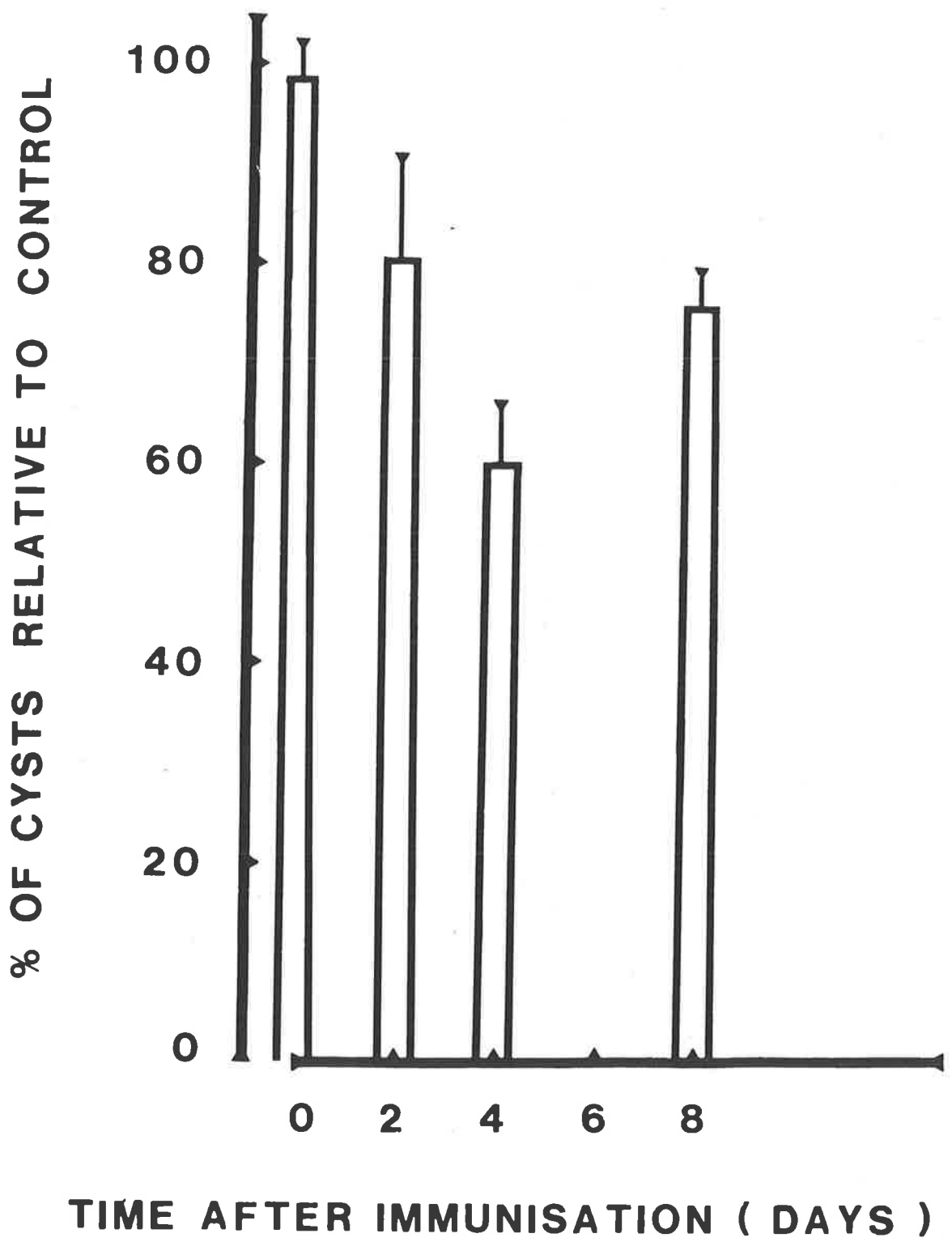
of cells. The tubes were incubated for a further 48 hours, and the contents of the tubes fed to mice, and cyst counts performed 6 days later. The number of cysts counted was expressed as a percentage of those observed in mice fed larvae from tubes containing larvae and immune serum in the absence of neutrophils. Neutrophils capable of impairing the infectivity of L3 in-vitro arise by day 4 following infection with S. enteritidis 11RX, indicating a rapid appearance of 'stimulated' neutrophils in a similar time span, as seen after infection with N. dubius.

5.11 EFFECT OF NEUTROPHILS FROM MICE IMMUNISED WITH KILLED VACCINES (KILLED L3 OR ADULT WORM EXTRACT) OF N. dubius ON THE INFECTIVITY OF L3 IN-VITRO

Killed larvae of N. dubius have in the past been unable to induce resistance against this parasite, whereas living vaccines have been highly successful (Chaicumpa, 1973). To assess whether these differences were due to an inability of killed vaccines to give rise to suitable effector cells, the following experiment was carried out.

Neutrophils were purified from the peripheral blood of mice, 7 days after a third dose given intra-venously at weekly intervals of either 2000 frozen-thawed exsheathed L3 (killed larvae), or 1.0 ml of a whole worm extract, prepared as out-lined in Chapter 2, section 2.6. Five mice from the killed larvae immunised group, were killed by cervical dislocation at this time, and the intestines removed and examined for adult worms. No adult worms were detected in the intestine of mice immunised with killed L3.

Eight groups of 5 tubes were set up, containing 200 exsheathed L3 in 0.2 ml of supplemented medium. To tubes in groups 2, 4 and 7 were added 0.05 ml of normal serum, whilst to groups 3, 5 and 8 were added 0.05 ml of immune serum, from the mice immune to N. dubius

After incubation at 37 C for 90 minutes 5 x 10^5 neutrophils from mice immunised with killed L3 were added to groups 4 and 5 in 0.2 ml of supplemented medium. To groups 6, 7 and 8 were added 5 x 10^5 neutrophils from mice immunised with adult worm extract in (a) medium alone, (b) normal serum or (c) immune serum in the absence of cells. The tubes were incubated for a further 48 hours, after which the contents were fed to mice and cysts counted 6 days later.

The data in Table 5.8 indicates that killed vaccines of N. dubius are capable of inducing 'stimulated' neutrophils. A living vaccine of L3 was not essential for the induction of neutrophils capable of killing exsheathed L3 in-vitro in the presence of immune or normal serum. Therefore, the inability of killed vaccines to protect mice in-vivo cannot be attributed to a lack of effector neutrophils, but may result from a humoral response deficient in protective antibodies. Ey (Personal communication) has demonstrated high titres of specific antibody as measured by ELISA against larval antigens, in the serum of killed L3 immunised mice.

5.12   ADHERENCE OF NEUTROPHILS FROM NORMAL MICE, AND MICE IMMUNE TO N. dubius INFECTION TO THE SURFACE OF EXSHEATHED L3

Neutrophils from N. dubius resistant mice, may differ from those of normal mice, in a number of ways. One aspect, which may differ is the density and/or specificity of Fc receptors on the cell surface. A higher density of Fc receptors on neutrophils from resistant mice, would allow for a higher binding efficiency to the parasite surface, and therefore an increased number of cells capable of damaging the larval cuticle. Alternatively ' stimulated ' neutrophils, Fc receptor specificity may be primarily directed against the immunoglobulin class in

TABLE 5.8

Effect of neutrophils from mice immunised with killed vaccines (killed L3 and adult worm extracts) of N. dubius on the infectivity of L3 in-vitro

Serum added	No cells	neutrophils added from mice immunised with :	
		Killed L3	Adult worm extract
None	(1) 200 ± 7	ND	(6) 184 ± 10
Normal serum	(2) 195 ± 7	(4) 146 ± 14	(7) 161 ± 13
Immune serum	(3) 184 ± 27	(5) 139 ± 19	(8) 155 ± 10

Figures in parenthesis represent group numbers

Group 2 vs. groups 4 and 7 $P < 0.05$

Group 3 vs. groups 5 and 8 $P < 0.05$

('U' test of Mann and Whitney)

which, the majority of anti-N. dubius antibody is found.

A precise analysis of the receptors of these cells would require their isolation and characterisation, however an indication of any differences in the receptors, may be obtained by a study of the adherence requirements of the cells from resistant and normal mice. In order to investigate this possibility, two series of 6 tubes were set up containing 500 exsheathed L3, incubated with fresh immune serum, or normal serum (0.05 ml) in VB^{2+}, VB^{2+}-EGTA, or VB^{2+}-EDTA. Control tubes contained exsheathed L3 in VB^{2+} only. The larvae were incubated for 90 minutes at 37°C to allow antibodies to bind and complement to be activated at the parasite surface. The larvae were washed twice with supplemented medium, and 10^6 neutrophils from N. dubius resistant or normal mice were added to the tubes. The tubes were further incubated for 60 minutes to allow cells to adhere to the larval surface. Following incubation, 30 larvae per tube were scored for the number of adherent cells as outlined in Chapter 4. The number of adherent cells, which were counted under the different conditions, Table 5.9, when neutrophils from resistant mice were used was similar to that reported in Chapter 4. Neutrophils from normal mice were no different in their behaviour and adhered to the surface of exsheathed L3 via C3/C3 receptor or via Fc/Fc receptor inter-actions.

However, a difference was observed in the number of cells adhering to the surface of larvae in the presence of antibody alone. Fewer neutrophils from normal mice were observed to adhere to the surface of L3 incubated in immune serum in the presence of EDTA, when compared with the number of neutrophils from resistant mice adhering under the same conditions. This may reflect an increase in the number of Fc receptors on the membrane of neutrophils 'stimulated' as a consequence of

TABLE 5.9

Adherence of neutrophils from normal mice, and mice immune to N. dubius infection to the surface of exsheathed L3.

Serum added in	N. dubius resistant mice	Normal mice
	Average number of adherent neutrophils / larva $\pm$ SD	
No serum	0	0
Immune serum VB^{2+}	80 $\pm$ 12	69 $\pm$ 9
Immune serum VB^{2+}- EGTA	76 $\pm$ 9	68 $\pm$ 8
Immune serum VB^{2+}- EDTA	51 $\pm$ 15	20 $\pm$ 9
Normal serum VB^{2+}	73 $\pm$ 9	67 $\pm$ 8
Normal serum VB^{2+}- EDTA	0	0

infection of mice by <u>N. dubius</u>.

5.13 ROLE OF ANTIBODY AND COMPLEMENT IN MEDIATING NEUTROPHIL AND EOSINOPHIL LARVICIDAL ACTIVITY

When only specific antibody was present in the assay systems described in this chapter (ie: heated immune serum), no killing by either eosinophils or neutrophils could be detected. Since one cannot exclude the possibility that the antibody was affected by heating the serum, it was therefore decided to use an immunoglobulin fraction from immune serum in the larvicidal assay, together with immune serum which had been depleted of complement activity by methylamine. Pangburn <u>et al</u>., (1981) reported, that methylamine inactivates C3, C4 and 2-macroglobulin, and is thought to be a milder way of inactivating complement than heating at 56°C for 30 minutes, since heating may even, at this temperature cause immunoglobulins to aggregate.

Mice were bled for serum, 10 days after a second immunising infection of <u>N. dubius</u>. An immunoglobulin fraction of the serum was obtained by addition of saturated ammonium sulphate to 50% saturation. In addition, 1.5 ml of the immune serum were incubated with 0.15 ml of 1M methylamine for 2 hours at 37°C, to inactivate complement, and then extensively dialysed in phosphate buffered saline containing Mg^{++} and Ca^{++}. A third proportion of the immune serum was kept on ice and used fresh in the assay. Normal serum was also collected and used either fresh, or after methylamine treatment. The same experimental protocol was used as previously described. Nine groups of 5 tubes were set up, each containing 200 exsheathed L3 in 0.2 ml of supplemented medium. To groups 3 and 4 were added 0.05 ml of immune serum (untreated), to groups 7 and 8, 0.05 ml of normal serum (untreated), to group 5, 0.05 ml of methylamine inactivated immune serum, while to group 6, 0.05 ml of the

TABLE 5.10

Role of antibody and complement in mediating neutrophil and eosinophil larvicidal activity in-vitro

	Cells added			
	Experiment 1		Experiment 2	
Serum added	None	Neutrophils	None	Eosinophils
	mean cyst counts $\pm$ SD			
None	(1) 172 $\pm$ 31	(2) 200 $\pm$ 22	143 $\pm$ 19	ND
Untreated IMS	(3) 163 $\pm$ 13	(4) 100 $\pm$ 38	132 $\pm$ 5	68 $\pm$ 18
Methylamine treated IMS	ND	(5) 162 $\pm$ 19	ND	123 $\pm$ 27
Immunoglobulin fraction from IMS	ND	(6) 196 $\pm$ 20	ND	133 $\pm$ 10
None	(1) 143 $\pm$ 19	ND	ND	ND
Untreated NMS	(7) 134 $\pm$ 6	(8) 66 $\pm$ 12	ND	ND
Methylamine treated NMS	ND	(9) 132 $\pm$ 10	ND	ND

Group 3 vs. group 4 $P<0.05$, Group 7 vs. group 8 $P<0.05$

Group 3 vs. groups 5 and 6 $P>0.05$ not significant

Group 7 vs. group 9 $P>0.05$ not significant

immunoglobulin fraction from immune serum. Finally to group 9, 0.05 ml of methylamine inactivated normal serum were added. After 90 minutes incubation at 37°C, 5×10^5 neutrophils were added to the larvae in groups 2, 4, 5, 6, 7, 9. Control groups contained larvae incubated in supplemented medium alone, and larvae incubated with fresh immune or normal serum.

After incubation at 37°C for 48 hours, the contents of the tubes were fed to mice, and cyst counts performed on day 6 (Table 5.10). The data indicates that neutrophils, in the presence of specific antibody alone, were unable to impair the infectivity of larvae in-vitro. Treatment of immune serum with methylamine to inactivate complement, or the use of a purified immunoglobulin fraction, resulted in comparable results with that obtained with heated immune serum in the assay, further confirming the inability of these cells to damage larvae, in the presence of antibody alone in-vitro.

The experiment was repeated with eosinophils, in the presence of fresh immune serum, methylamine inactivated serum and purified immunoglobulin from N. dubius immune serum (Table 5.10). The results were similar to those obtained using neutrophils.

5.14 CONCLUSIONS

The role of eosinophils and neutrophils in immunity to N. dubius has been examined in this study, by assessing the effect of these cells on the infective third-stage larvae in-vitro. Eosinophil and neutrophil enriched cell populations were isolated by density centrifugation from the peripheral blood of mice infected with this parasite.

Eosinophils and neutrophils were found to be more efficient at inactivating the exsheathed larvae of N. dubius in association with antibody and complement together (immune serum), as compared with complement alone (normal serum). It should be noted,

however, that any conclusions concerning the role of antibody in this result, depends on the level of complement in IMS being equivalent to that in normal serum. When only specific antibody was present in the assay system (heated immune serum, methyl-amine inactivated immune serum or a purified immunoglobulin fraction of immune serum), no killing by either eosinophils or neutrophils could be detected.

Incani and McLaren (1981) demonstrated that although rat neutrophils could adhere to the surface of Schistosoma mansoni in the presence of heated immune rat serum, they would detach after 6 hours without causing any mortality. Similarly, it was observed in the present study, that the cells would adhere to the surface of the parasite via specific antibody alone. However, by 24 hours in culture the cells had detached, and no impairment to the infectivity of the larvae resulted. The ability of neutrophils and to some extent eosinophils from immunised mice to inactivate the parasite in the presence of complement alone is probably related to the ability of the larvae to activate complement via the alternate pathway, binding of the cells to the larvae, then taking place via C3b which can be visualised on the parasite surface, using fluorescein-labelled anti-C3 antibody (Prowse, Ey and Jenkin 1979; Ey, personal communication).

An important point arising from this study, was that neutrophils from normal mice, were unable to damage N. dubius larvae in-vitro even in the presence of antibody and/or complement, conditions under which neutrophils from immune mice were highly effective. It seems therefore, that neutrophils from mice infected with N. dubius, are altered in their activity in some way.

Studies with human neutrophils, have indicated that neutrophils acquire an enhanced capacity to phagocytose and kill

Serratia marcescens after exposure to supernatants of activated
human mononuclear cells (Cross and Lowell, 1978). Human
neutrophils have also been reported to be stimulated in
exocytosis, migration, superoxide production and aggregation by
exposure to a platelet-activating factor (James et al., 1981).
These findings clearly indicate that neutrophils can undergo a
change in activity as a consequence of infection.

Whether this alteration of neutrophil activity requires the
participation of accessory cells, as does the activation of
macrophages is at present not known. However, what is clear, is
that the appearance of these cells with larvicidal properties
is not a specific response to infection with N. dubius, since
they also appear in the peripheral blood of mice, which have been
infected with Salmonella enteritidis 11RX, killed larvae, or
adult worm extract. These altered cells are present in the
peripheral blood, 4 days after a primary infection and appear
to be present up to at least 6 weeks after a secondary infection.

Thus, it is clear from in-vitro studies that three
phagocytic cell types may play a role in immunity to this
infection. Previous investigations have implicated activated
macrophages (Chaicumpa & Jenkin, 1978) and the present study
implicates, both the neutrophil and eosinophil. The in-vitro
larvicidal activity of peritoneal cells from S. enteritidis 11RX
immunised mice as reported by Chaicumpa and Jenkin, (1978),
cannot be solely attributed to 'activated' macrophages. Since
S. enteritidis 11RX infection also induced 'stimulated'
neutrophils, the presence of these cells in the peritoneal cell
population may also contribute to the larvicidal activity
reported.

CHAPTER 6.

THE ROLE OF NEUTROPHILS IN-VIVO IN IMMUNITY

TO N. dubius INFECTIONS IN MICE

6.1   INTRODUCTION

Data in the previous Chapter, indicated that purified eosinophils and neutrophils reduced the infectivity of N. dubius when they were incubated with the larvae in-vitro, in the presence of antibody and/or complement, but not in the presence of antibody alone. Furthermore, neutrophils from immune mice were shown to become 'stimulated' in their larvicidal activity as a consequence of infection, such that they were able to kill larvae in-vitro under the above conditions, whereas neutrophils from normal mice were ineffective. This enhanced larvicidal activity was shown to arise within four days, after a primar infection with N. dubius. The aim of the experiments outlined in the present Chapter, were to assess in-vivo the significance of these cells in immunity. The immune sera used throughout these experiments were collected from mice 10 days after a second immunising infection of 200 L3 iv.

6.2   EFFECT OF SERUM TRANSFER ON THE RESISTANCE OF MICE
       TO REINFECTION WITH N. dubius FOUR DAYS AFTER A
       PRIMARY IMMUNISING INFECTION

The results in the previous Chapter, showed that neutrophils with in-vitro larvicidal activity could be purified from the peripheral blood of mice, as early as four days following a primary infection with N. dubius. However, despite the appearance of these cells, mice which have been infected for four days, show no significant resistance to a second challenge infection of N. dubius larvae, given 4 days after the first immunising infection (Prowse et al., 1978a). This might imply, that there was, either insufficient antibody available at this early stage of the infection, to direct the attack of effector cells, or that 'stimulated' neutrophils in-vivo were ineffective against N. dubius as compared to the in-vitro situation. The

following experiment was carried out to clarify this situation.

Ten groups of 6 mice were immunised with 200 L3 intra-venously on day 0 to induce 'stimulated' neutrophils. The mice were then rechallenged on day 4 with 200 L3 intra-venously. It should be noted, that there is no difference in the number of cysts detected in the intestine of mice, after either an oral or intra-venous challenge with L3 (Chaicumpa et al., 1977). The mice within a given group, were further injected with either 1.0 ml of immune serum intra-peritoneally on the day of challenge or 1,2,3 and 4 days later ie: days 5,6,7 and 8 post-challenge. Two further groups of mice were given either 1.0 ml of an IgG (15 mg/ml) or an IgM (0.4 mg/ml) immunoglobulin fraction from immune serum, on the day of challenge. The IgG and IgM immunoglobulin fractions were prepared from immune serum by Sephadex G200 chromatography as outlined in Materials and Methods(Section 2.13). The concentrations of IgG and IgM transferred in the experiment, were equivalent to the levels of these immunoglobulin classes found in immune serum, collected from mice 10 days after a second immunising infection of 200 L3 iv. Mice in the control groups were given either 1.0 ml of saline, or 1.0 ml of normal serum ip. on the day of rechallenge (day 4). Resistance was assessed by counting cysts in the intestinal wall 6 days after the challenge infection. In the case of mice, which had received 1.0 ml of immune serum on day 8, adult worms in the intestine were counted 10 days after the challenge infection. The reason for this, was that serum was given to the mice at a time when larvae from the challenge infection were already migrating as young worms to the intestine. The cyst counts in the other groups, represent the number of surviving larvae from the challenge infection, since larvae from the primary infection were at the adult worm stage at the time that the cysts were counted. In contrast, where adult

FIGURE 6.1

The effect of serum transfer on the resistance of once-
infected mice to a challenge dose of N.dubius larvae.
LACA mice were injected ip. with one, 1.0 ml dose of
normal or immune serum at various times (4-8 days) after
they had received an 'immunising' primary dose of 200 L3
iv.All the mice were challenged with 200 L3 iv. on day 4
They were killed on day 10(= 6 days post-challenge) for
cyst counts or on day 14 for adult worm counts. The latter
comprised the cumulative dose of worms from both infections.
NS.= not significant ($p > 0.05$)

☐ No serum transferred ▨ Normal serum ■ Immune serum
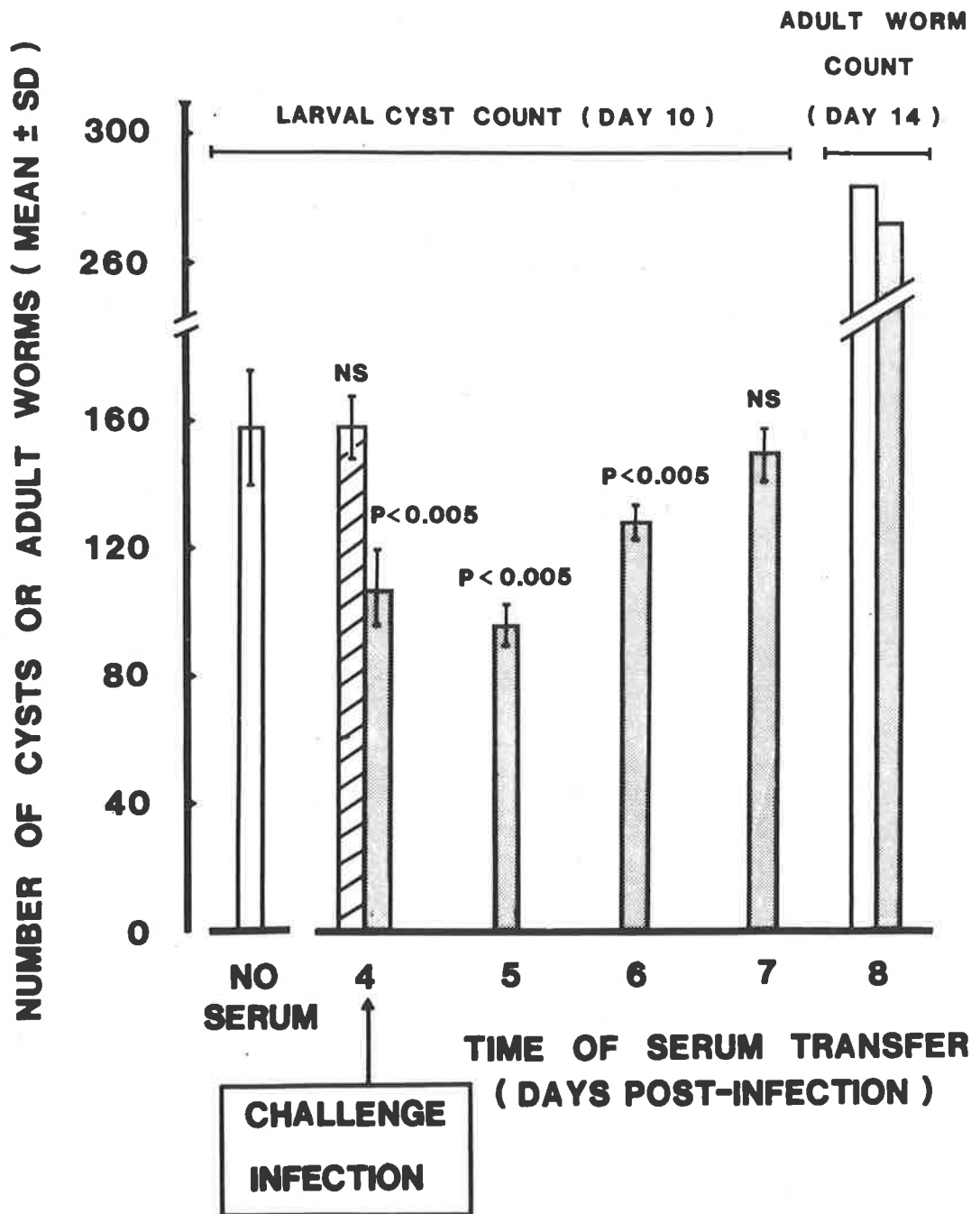
TABLE 6.1

The capacity of an IgG or an IgM immunoglobulin fraction from
immune serum to confer resistance to pre-infected mice against
a second infection with N. dubius L3

Serum transferred	Cyst count (mean $\pm$ SD)
Immune serum	107 ± 13 *
Normal serum	158 ± 9
IgG fraction of immune serum	84 ± 13 *
IgM fraction of immune serum	108 ± 10 *
No serum	158 ± 11

* P values compared with control group given normal serum
  where $P < 0.05$ ('U' test of Mann and Whitney)

worms were counted, the results are representative of a count of worms from both the immunising infection, as well as the challenge infection.

The data in Fig. 6.1 indicates that mice which were challenged 4 days after a primary immunising infection, were partially resistant to the challenge dose of larvae, provided they were also injected with immune serum, at the time of challenge, or within 24-48 hours post-challenge. Maximum protection resulted, if serum was transferred within 24 hours post-challenge. Transfer of immune serum after 72 hours, had no effect on the resistance of mice to rechallenge.

Both the IgG and the IgM immunoglobulin fractions of immune serum, when given to mice at the time of rechallenge (4 days after a primary infection) also conferred resistance (Table 6.1). The IgG fraction was slightly more efficient than the IgM fraction in conferring protection (Table 6.1). $P < 0.005$

6.3 PASSIVE TRANSFER OF RESISTANCE TO PRIMARY INFECTED MICE: TITRATION OF IMMUNE SERUM

Various dilutions of immune serum were assayed for their ability to passively transfer immunity to primary immunised mice. Six groups of 6 mice were immunised with 200 larvae intravenously on day 0. Four days later, the mice were given a second challenge dose of 200 larvae iv. Varying dilutions of immune serum (1.0 ml) from N. dubius infected mice, as outlined in Fig. 6.2 were injected intra-peritoneally into mice in 5 of the groups at the time of challenge ie: day 4. A control group of mice received 1.0 ml of saline ip. at the same time ie: 4 days after the primary infection. All mice were killed by cervical dislocation on day 10 and cysts counted in the intestinal wall. The data in Fig. 6.2 shows that the degree of protection decreased as the dilution of antibody was increased, such that at a dilution of $1/16$ no protection resulted. Low

FIGURE 6.2

Titration of immune serum for its capacity to protect mice against re-infection with N. dubius. Groups of LACA mice were infected with N. dubius. Four days later, they were re-infected with an iv. challenge dose of 200 L3 and simultaneously injected with 1.0ml of immune serum ip., or serum diluted in saline. The mice were killed on day 10 for cyst counts (6 days post-challenge). Significance was determined by the 'U' test of Mann and Whitney. Experimental groups were compared with the control group given no serum.
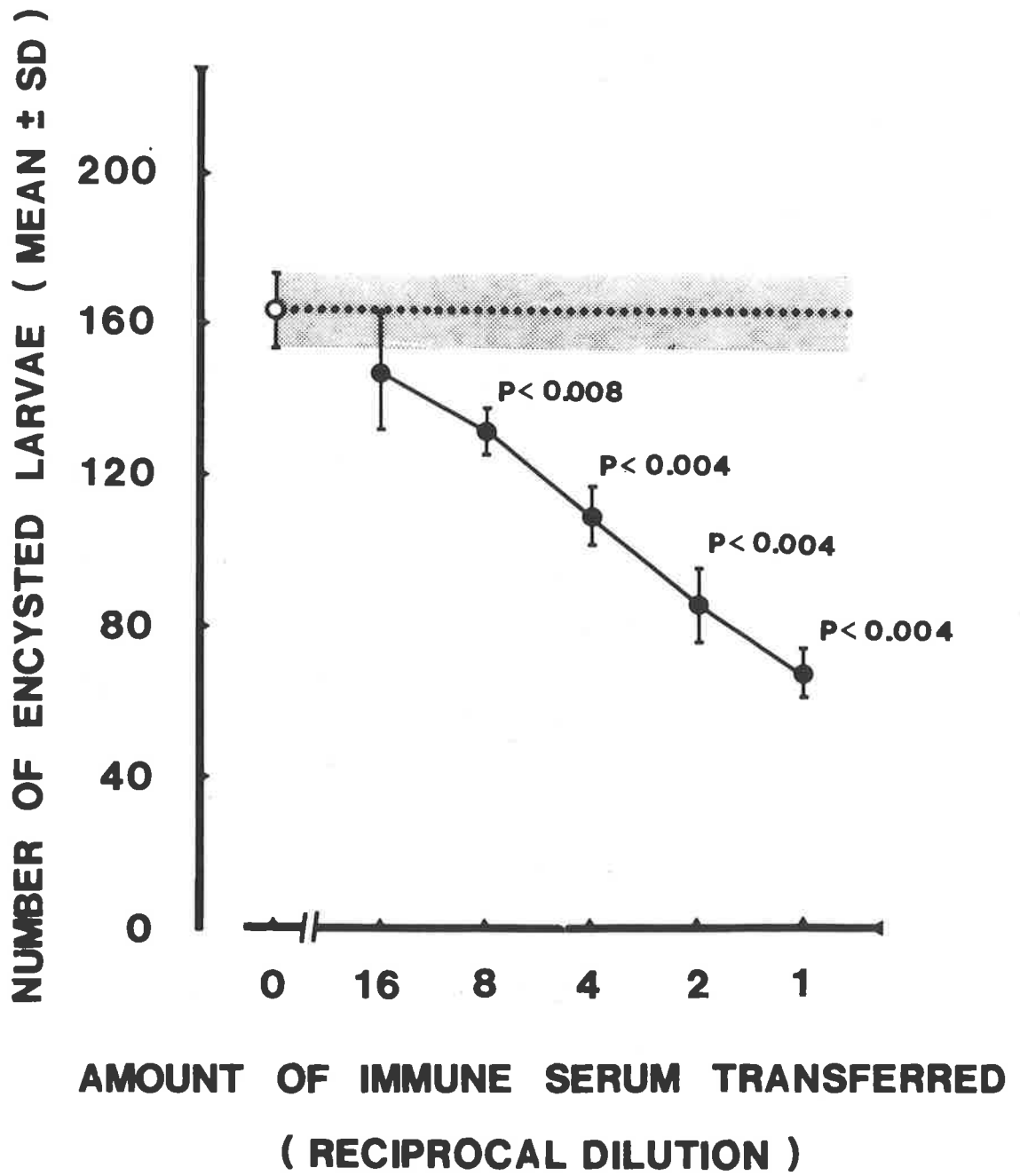
levels of serum however still conferred protection, as serum dilutions of 1/4 equivalent to 0.25 ml of immune serum resulted in 34% immunity as measured by comparing cyst counts from mice given serum at this dilution, with control mice given saline only. It has previously been shown that passive transfer of immune serum to naive mice doesn't lead to increased resistance to N. dubius, this control group was therefore not included in this experiment (Chaicumpa, Jenkin and Rowley, 1976).

6.4 EFFECT OF PERITONEAL WASHOUT CELLS ON THE INFECTIVITY OF LARVAE, FOUR DAYS AFTER A PRIMARY CHALLENGE INFECTION OF N. dubius

The previous results implicated the neutrophil as the cell type responsible for the immunity seen following transfer of immune serum to primary immunised mice in the early stages of infection. 'Stimulated' neutrophils can be purified from the blood of mice 4 days after a primary infection of N. dubius. However, macrophages which increase in number as a consequence of infection, are also present at this stage of infection (Prowse et al., 1978a), but whether these cells are in an 'activated' state and therefore capable of killing L3 in-vitro or in-vivo is unclear.

In order to clarify this situation, purified blood neutrophils, as well as peritoneal washout cells, which were predominantly macrophages, from mice 4 days after primary infection were compared for their in-vitro larvicidal activity by the method outlined in Materials and Methods, (Section 2.11). To two experimental groups of 5 tubes containing 200 exsheathed L3 in 0.2 ml of supplemented medium, were added 0.05 ml of immune serum. A further control group of 5 tubes also received 0.05 ml of immune serum, while another control group contained 200 L3 in medium alone. The tubes were then incubated for 90 minutes at 37°C. Neutrophils (10^5) purified from the blood of

TABLE 6.2

In-vitro larvicidal activity of peritoneal washout cells and peripheral blood neutrophils collected from LACA mice 4 days after a primary infection with N. dubius

Cells added (10^5 per tube)	Immune serum	Cyst count (mean $\pm$ SD)
None	-	193 $\pm$ 18
None*	+	206 $\pm$ 9
Peritoneal cells	+	191 $\pm$ 3 (P=0.2)
Neutrophils	+	136 $\pm$ 34 (P=0.004)

Larvae were incubated for 48 hours in-vitro with cells and immune serum as indicated in the text, then they were given per os to normal mice to assess infectivity.

P values determined by 'U' test of Mann and Whitney

Experimental groups were compared for significance with the control group labelled *.

mice 4 days after a primary infection of N. dubius in 0.2 ml
of supplemented medium, were added to the tubes in one experi-
mental group. Peritoneal washout cells (10^5) from the same
group of mice, were then added to tubes in the other group.
After the addition of cells, the tubes were incubated for 48
hours at 37°C. The contents were then fed to mice and cyst
counts performed 6 days later.

As can be seen from the data in Table 6.2, peritoneal cells,
4 days after a primary infection of N. dubius, show no larvicidal
activity in-vitro against N. dubius, whereas the purified
neutrophils from these mice, reduced larval infectivity by
approximately 30%. The lower number of 10^5 peritoneal cells
was chosen, because the level of contaminating neutrophils (5%)
was below 10^4. Neutrophils at this concentration have been
shown previously, to be inactive against larvae in-vitro
(Chapter 5). 'Activated' macrophages, at this level are highly
active in an in-vitro larvicidal assay (Desakorn, Personal
communication). Therefore, the effector cells responsible for
resistance in mice 4 days after infection, do not appear to be
macrophages.

6.5   IN-VITRO LARVICIDAL ACTIVITY OF NEUTROPHILS FROM
        N. dubius RESISTANT AND NORMAL (C57/BL x BALB/c
        F1 HYBRID MICE

The LACA strain of mice used in the previous experiments
were an outbred strain, and therefore unsuitable for use in
cell transfer experiments, for this reason in the following
experiments (C57/BL x BALB/c) F1 hybrid mice were used.
Initially, studies were carried out to determine whether neutro-
phils from the peripheral blood of F1 mice were capable of
killing exsheathed L3 in-vitro. F1 hybrid mice were given two
immunising infections of 200 L3 iv. at 14 day intervals,
neutrophils were purified from the blood of these mice 10 days

TABLE 6.3

In-vitro larvicidal activity of neutrophils purified from
the peripheral blood of N. dubius resistant and normal
(C57/BL x BALB/c) Fl hybrid mice

Serum added	Cells from:		
	No cells	Normal mice	Resistant mice
	mean cyst counts $\pm$ SD		
None	(1) 134 ± 12	ND	ND
Immune serum	(2) 132 ± 3	(3) 121 ± 6	(4) 61 ± 5
Normal serum	(5) 129 ± 4	(6) 130 ± 4	(7) 94 ± 10

1×10^6 neutrophils added per tube

 Figures in parenthesis represent group numbers

Groups 2 vs. 4 and 5 vs. 7 $P < 0.05$

Groups 2 vs. 3 and 5 vs. 7 $P > 0.05$ not significant

after the second immunising infection. Neutrophils were also purified from the blood of normal F1 mice.

To 7 groups of 5 tubes containing 200 exsheathed L3 in 0.2 ml of supplemented medium, were added either 0.05 ml of immune serum to groups 2,3 and 4, or 0.05 ml of normal serum to tubes in groups 5,6 and 7. The tubes were incubated at 37°C for 90 minutes to allow antibody to bind to the parasite surface and/or complement activation. Neutrophils, (10^6) from normal or resistant mice were then added in 0.2 ml of supplemented medium to tubes in groups 3 and 6 or 4 and 7 respectively. The tubes were incubated for a further 48 hours, after which the contents were fed to mice and cysts counted 6 days later.

Data in Table 6.3 indicates that neutrophils from immunised F1 hybrid mice were capable of impairing the infectivity of L3 in-vitro in the presence of antibody and/or complement. However, as in the case of LACA mice, neutrophils from normal mice were ineffective.

6.6 THE EFFECT OF THE TRANSFER OF NEUTROPHILS AND EOSINOPHILS FROM RESISTANT MICE TO NAIVE MICE ON THEIR RESISTANCE TO INFECTION WITH N. dubius

To assess the capacity of neutrophils and eosinophils to protect mice (in-vivo) against infection with N. dubius, neutrophils and also eosinophils were purified from the blood of immune F1 mice. Mice were immunised with 200 L3 iv. on day 0 and again on day 14, and some bled for polymorphonuclear cells on day 24, and others on day 25. Six groups of 6 mice were each challenged with 200 larvae iv. on day 0. The mice in group 2 and 4 were injected iv. with 10^6 neutrophils in 0.2 ml of basic medium on day 0, and again on day 1. Similarly, mice in groups 5 and 6 were injected with 10^6 eosinophils on the same days. Control mice in group 1 received medium only, and mice in groups 3,4 and 6 were given 0.5 ml of immune serum ip. on days -1,0 and 1.

TABLE 6.4

The effect of the transfer of neutrophils and eosinophils
from resistant mice to naive mice on their resistance to
infection with N. dubius

Group number	Treatment of mice	cyst counts (mean ± SD)
1	Nil	154 ± 9
2	Neutrophil transfer 10^6 per mouse	155 ± 9
3	Immune serum only	150 ± 10
4	Neutrophil transfer plus immune serum	101 ± 9
5	Eosinophil transfer 10^6 per mouse	153 ± 11
6	Eosinophil transfer plus immune serum	148 ± 14

Cells were transferred on the day of challenge (day 0) and
again on day 1. Serum (0.5 ml) was transferred on days -1, 0 and 1
Group 1 vs. group 4, and group 2 and 3 vs. group 4 $P < 0.05$
Group 1 vs. groups 2 and 3 or 5 and 6 $P > 0.05$ not significant

Immunity was assessed by counting cysts 5 days later.

As shown in Table 6.4, partial resistance could be transferred by neutrophils from immune mice, but only if immune serum was also given. Eosinophils from resistant mice were ineffective in transferring immunity, even in the presence of immune serum. Since immune serum alone conferred no resistance upon transfer, it is unlikely that the immune serum given in the above experiment resulted in activation of host cells by such factors as lymphokines or colony stimulating factors in the serum. Furthermore, a cell population containing 70% lymphocytes and 30% monocytes from immune mice were shown to be inactive against the larvae in-vitro, (Chapter 5). Since only 2% ± 1% contaminating lymphocytes were present in the transferred cell population in this experiment (98% ± 4% neutrophils), it is unlikely that the low level of contaminating cells could have been responsible for the protection observed. However, one cannot exclude the possibility that factors may be released from the contaminating lymphocytes, which may activate host cells other than neutrophils. However, experiments by Behnke and Parish, (1981) indicates that a higher number of lymphocytes would be required to transfer immunity, since in their experiments 10^6 Mesenteric lymphnode cells were needed to confer protection.

6.7   CONCLUSIONS

Neutrophils with an enhanced in-vitro larvicidal activity can be purified from the peripheral blood of mice, as early as four days after a primary infection. Mice which were rechallenged 4 days after primary infection with N. dubius larvae showed an enhanced resistance to the second challenge infection provided immune serum, was also passively transferred at the time of challenge or within 48 hours of the second challenge infection.

Since 'activated' macrophages were not present at this early stage of infection, it could be assumed that this enhanced resistance to the second challenge resulted from the effect of 'stimulated' neutrophils, along with an increase in specific antibody. Transfer of immune serum 72 hours or later, after the second challenge conferred no resistance to mice, even though 'stimulated' neutrophils could be purified from the circulation. These cells appear to act as effector cells against the early stages in the N. dubius life cycle in-vivo in conjunction with specific antibody and/or complement. Transfer of either an IgG or an IgM fraction of immune serum, at a concentration equivalent to that found in whole immune serum, conferred resistance to mice which had 'stimulated' neutrophils. Mouse neutrophils have been shown to have receptors for IgG, but not IgM coated SRBC (Lopez et al., 1981). Therefore, resistance transferred to mice via the IgM fraction of immune serum, may be due to activation of complement, which could result in increased adherence of neutrophils via C3 receptors to the larval surface.

To further assess whether neutrophils were an effector cell involved in immunity to N. dubius in-vivo a passive transfer of 'stimulated' neutrophils to naive mice was carried out. Transfer of neutrophils along with immune serum conferred a partial resistance to challenge with N. dubius, both cells and serum were required together; neither alone had an effect on immunity. Eosinophils were ineffective in transfer.

From the data, it appears that neutrophils may be involved during the initial phase of infection with N. dubius and act in conjunction with specific antibody in killing the early stages in the life cycle.

CHAPTER 7.

SUPPRESSION IN-VIVO OF IMMUNITY TO N. dubius IN

MICE BY SELECTIVE DEPLETION OF NEUTROPHILS AND

EOSINOPHILS WITH SPECIFIC MONOCLONAL ANTIBODIES

7.1   INTRODUCTION

Eosinophils and neutrophils in-vitro will adhere to and kill the exsheathed third stage larvae of N. dubius in the presence of antibody and complement alone. Furthermore, neutrophils were required to be in a 'stimulated' state before detectable damage to the larvae, as measured by a loss in infectivity occurred. This requirement was based on the finding that neutrophils from normal mice were totally inactive against the larvae, under the same serum conditions in which neutrophils from immune mice were able to damage and kill larvae. To assess further the role of eosinophils and neutrophils as effector cells in determining resistance against N. dubius infections, experiments were carried out in-vivo to investigate what affect depletion of these cells by specific monoclonal antibodies had on resistance to reinfection.

7.2   DEPLETION OF POLYMORPHONUCLEAR CELLS FROM N. dubius RESISTANT MICE BY TREATMENT WITH MONOCLONAL ANTI-GRANULOCYTE ANTIBODIES

The monoclonal antibodies used in these experiments were NIMP - R6 which bound preferentially to murine eosinophils and NIMP - R10 which bound to mature non-dividing neutrophils, as well as eosinophils. The properties of these two monoclonal antibodies have been reported by Lopez, Strath and Sanderson, (1983).

To ensure that NIMP - R10 and NIMP - R6 treatment of mice specifically depleted polymorphonuclear cells and had no effect on other cell types, total and differential cell counts were carried out on the blood and peritoneal washouts of mice treated with the above monoclonal antibodies. A group of 40 mice received two infections of 200 L3 intra-venously at 14 day intervals, and a third infection, 10 days after the second. The third dose of L3 correlated with the time of the challenge

infections described in the following experiments, and was given to ensure that a decrease in polymorphonuclear cells as a consequence of treatment of mice by specific monoclonal antibody, was not altered as a result of the challenge infection. Twenty of these immune mice were injected with 1.0 ml of a ^{1}/50 dilution of NIMP - R10 ascitic fluid on days 23,24 and 25 of infection ie: one day prior to, at the time of, and 1 day after challenge. The remaining 20 mice were injected with 1.0 ml of a ^{1}/25 dilution of NIMP - R6 ascitic fluid on the same days as above. Total and differential leukocyte counts of peripheral blood and peritoneal washouts were carried out on 5 of the mice on day 23, prior to treatment with the monoclonal antibodies. Leukocyte counts were also done on days 24 and 25, ie: 24 hours after each treatment with either NIMP - R10 or NIMP - R6 as well as on day 28, ie: 72 hours after the last injection of monoclonal antibody. Leukocyte counts were also carried out on 5 naive mice at each time point, (Table 7.1 and Table 7.2).

NIMP - R10 treatment of mice caused a significant fall (50%) in the number of neutrophils in both the circulation and peritoneal cavity during the first 2-3 days of treatment, compared to the number observed in untreated control mice.

There was no detectable change in the numbers of lymphocytes, or eosinophils in either the peripheral blood or peritoneal cavity, but there was a slight decrease in macrophage levels in the peritoneal washouts on day 25 (18%) and day 28 (19%). Treatment of mice with NIMP - R10 caused a drop of approximately 35% in total leukocyte numbers in the blood, but had little effect on peritoneal cells, which were predominately macrophages. NIMP - R10 appeared to selectively deplete neutrophils from these mice. A similar result was obtained when NIMP - R10 was used at a ^{1}/100 dilution.

TABLE 7.1

The effect of NIMP-R10 and NIMP-R6 treatment on the differential
and total blood leukocyte counts of mice infected with <u>N. dubius</u>

Type of cell	Treatment of mice	Leukocyte composition on			
		Day23	Day24	Day25	Day28
Macrophage (%)	None	ND	18 ± 6	16 ± 4	9 ± 10
	NIMP-R10	21 ± 6	19 ± 4	15 ± 4	17 ± 0
	NIMP-R6	ND	21 ± 3	13 ± 3	8 ± 3
Lymphocyte (%)	None	ND	53 ± 8	63 ± 6	55 ± 10
	NIMP-R10	$47 - 2$	62 ± 7	68 ± 3	69 ± 8
	NIMP-R6	ND	50 ± 9	50 ± 6	61 ± 10
Neutrophil (%)	None	ND	22 ± 5	14 ± 4	25 ± 9
	NIMP-R10	28 ± 5	10 ± 5	7 ± 5	8 ± 3
	NIMP-R6	ND	24 ± 9	34 ± 8	29 ± 6
Eosinophil (%)	None	ND	8 ± 5	7 ± 2	11 ± 3
	NIMP-R10	4 ± 1	9 ± 7	10 ± 4	6 ± 1
	NIMP-R6	4 ± 1	5 ± 4	3 ± 1	2 ± 1
Total leukocytes (x 10^6)	None	ND	5 ± 1	5 ± 2	5 ± 1
	NIMP-R10	5 ± 1	5 ± 2	3 ± 1	3 ± 1
	NIMP-R6	ND	12 ± 4	10 ± 1	5 ± 1

Mice were bled on day 23(prior to treatment with NIMP-R10 or
NIMP-R6)and days 24 ,25 (24 hours after each treatment with
NIMP-R10 or NIMP-R6) as well as 72 hours after the last injection
of monoclonal antibody(day 28).

All mice were given 200 L3 iv. on days 0,14 and 24. Those treated
with antibody were injected on days 23, 24 and 25.

ND = not done

TABLE 7.2

The effect of NIMP-R10 and NIMP-R6 treatment on the differential and total leukocyte counts of peritoneal washouts from mice infected with N. dubius

Type of cell	Treatment of mice	Leukocyte composition of			
		Day23	Day24	Day25	Day28
Macrophage	None	ND	78 ± 8	78 ± 10	80 ± 6
(%)	NIMP-R10	81 ± 5	72 ± 8	64 ± 11	65 ± 12
	NIMP-R6	ND	86 ± 6	84 ± 6	84 ± 3
Lymphocyte	None	ND	4 ± 2	8 ± 4	13 ± 2
(%)	NIMP-R10	4 ± 3	14 ± 4	17 ± 7	20 ± 15
	NIMP-R6	ND	5 ± 2	6 ± 3	9 ± 2
Neutrophil	None	ND	4 ± 3	4 ± 2	ND
(%)	NIMP-R10	1 ± 1	1 ± 1	2 ± 1	1 ± 1
	NIMP-R6	ND	3 ± 0	4 ± 1	ND
Eosinophil	None	ND	14 ± 4	10 ± 6	7 ± 4
(%)	NIMP-R10	14 ± 3	13 ± 3	17 ± 7	14 ± 6
	NIMP-R6	ND	6 ± 6	6 ± 3	8 ± 4
Total leukocytes	None	ND	9 ± 3	6 ± 2	10 ± 2
(x 10^6)	NIMP-R10	14 ± 4	9 ± 4	13 ± 1	10 ± 2
	NIMP-R6	ND	12 ± 5	10 ± 1	8 ± 1

Mice were bled on day 23 (prior to treatment with NIMP-R10 or NIMP-R6) and days 24, 25 (24 hours after each treatment with NIMP-R10 or NIMP-R6), as well as 72 hours after the last injection of monoclonal anibody (day28).

All mice were given 200 L3 iv. on days 0,14 and 24. Those treated with antibody were injected on days 23, 24 and 25.

ND = not done

Mice treated with NIMP - R6 showed no significant changes in neutrophil levels in either the blood or peritoneal washouts, nor did they show a reduction in macrophage or lymphocyte levels. However, blood eosinophil levels were reduced by 40% on day 24 and by at least 60% on day 25 and 28. The number of eosinophils in peritoneal washouts also were reduced by NIMP - R6 treatment, although the effect was less pronounced, and not as persistent as that seen in the blood (eosinophil levels on day 28 were the same as in the control mice).

7.3 SUPPRESSION OF EARLY IMMUNITY TO N. dubius IN PRIMARY IMMUNISED MICE BY NIMP - R10 ASCITIC FLUID

The data in the previous Chapter, showed that mice given an immunising infection of L3 were partially protected against a challenge infection of 200 L3 given 4 days later, providing immune serum was also injected at the time of challenge. Normal mice given immune serum, show no resistance to challenge by N. dubius, which indicates that mice, 4 days after a primary immunising infection of 200 L3, have effector cells expressing larvicidal activity, which are not present in normal mice. To assess the significance of neutrophils in this immunity during the early stages of infection by N. dubius, 3 groups of 5 mice were given 200 L3 iv. on day 0 and were then challenged with 200 L3 4 days later. Mice in groups 3 and 4 received 1.0 ml of immune serum ip. at the time of challenge (day 4). In addition, mice in group 3 were given 1.0 ml of a ^{1}/100 dilution of NIMP - R10 ascitic fluid on days 3,4 and 5, ie: one day prior to, at the time of challenge and one day later. The control groups 1 and 2 consisted of naive mice or primary infected mice respectively challenged with 200 L3. Resistance to the challenge infection was assessed by counting encysted larvae on day 10.

The data in Table 7.3 shows that mice, which were challenged 4 days after a primary immunising infection of L3 showed

TABLE 7.3

Suppression of early immunity to <u>N. dubius</u> in primary immunised mice by treatment with NIMP-R10 ascitic fluid

Group	Mouse status at challenge	Immune serum	NIMP-R10 treatment	mean cyst count $\pm$ SD Expt. 1	Expt. 2
1	Normal	-	-	94 ± 7	115 ± 4
2	Infected	-	-	103 ± 3	99 ± 11
3	Infected	+	-	26 ± 3	42 ± 6
4	Infected	+	+	117 ± 12	112 ± 8

All mice were challenged on day 0 and killed on day 6 for intestinal cyst counts. Infected mice were injected iv. with 200 L3 4 days prior to challenge, to generate effector cells. Immune serum (1.0 ml ip.) on day 0 and NIMP-R10 antibody were given as indicated in the text.

Group 3 vs. groups 2 or 1 $<$P 0.05, Group 4 vs. group 3 P$<$0.05

Group 4 vs. groups 2 and 1, P$>$0.05 not significant

('U' test of Mann and Whitney)

increased resistance to the challenge infection, provided immune serum was also transferred. However, if mice were given NIMP - R10 to deplete neutrophils, even though they had received immune serum passively, immunity was completely suppressed, which suggests that neutrophils were a major effector cell in the early stages of immunity to N. dubius.

7.4 SUPPRESSION OF THE LATER IMMUNITY TO N. dubius IN PRIMARY IMMUNISED MICE BY NIMP - R10 AND NIMP - R6 TREATMENT

Mice 10 days after a primary immunising infection of N. dubius, kill up to 50% of a second challenge infection. To asses the importance of neutrophils in this resistance, 3 groups of 5 mice were given an immunising infection of 200 L3 iv. on day 0, and were then challenged with 200 L3 iv. 10 days later. Mice in group 2 were given 1.0 ml of a ^{1}/100 dilution of NIMP - R10 ascitic fluid on days 9,10 and 11 ie: one day prior to, at the time of challenge and one day later. Mice in group 3 received 1.0 ml of a ^{1}/25 dilution of NIMP - R6 ascitic fluid on the same days. Mice in control groups 1 and 4 consisted of primary immunised mice, or naive mice challenged with 200 L3 respectively. Resistance to the challenge infection was assessed by counting encysted larvae 6 days later.

The data in Table 7.4 shows that immunity to mice 10 days after a primary infection of N. dubius was only partially suppressed by NIMP - R10 depletion of neutrophils. Since these mice had a partial resistance to rechallenge, unlike mice treated with NIMP - R10 four days after a primary immunising infection, it implys that another cell type, other than the neutrophil may be important in determining resistance at this stage of the infection. No circulating eosinophils can be detected following a primary infection of N. dubius, although they may be found in the bone marrow (Prowse, Ey and Jenkin, 1978a). Therefore,

TABLE 7.4

Suppression of the later immunity to N. dubius in primary
immunised mice by treatment with NIMP-R10 and NIMP-R6
ascitic fluid

Group	Mouse status at challenge	Monoclonal antibody treatment	mean cyst count $\pm$ SD	
			Expt. 1	Expt. 2
1	Infected	None	42 ± 15	50 ± 10
2	Infected	NIMP-R10	97 ± 15	83 ± 11
3	Infected	NIMP-R6	ND	55 ± 7
4	Normal	None	146 ± 11	136 ± 12

All mice were challenged on day 0 and killed on day 6 for
intestinal cyst counts. Infected mice were injected iv. with
200L3 10 days prior to challenge, to induce partial immunity.

Group 3 vs. group 2 ; group 3 vs. 1 (both expt.) P<0.05
Group 4 vs. 2, not significant ; group 4 vs. 1 P<0.05

treatment of mice with NIMP - R6, which depletes circulating
eosinophils would be expected to have no affect on immunity to
a challenge infection of 200 L3, and as the data in Table 7.4
indicates the result was as expected.

7.5   SUPPRESSION OF IMMUNITY TO N. dubius IN SECONDARY
       IMMUNISED MICE BY NIMP - R10 AND NIMP - R6 TREATMENT

LACA mice given two immunising infections of N. dubius show
almost complete resistance to rechallenge with L3. To investi-
gate the affect of the depletion of eosinophils and neutrophils
in secondary immunised mice on their resistance, the following
experiment was carried out. Four groups of 5 mice received two
immunising infections of 200 L3 iv. at 14 day intervals, the
mice were then challenged with 200 L3, 10 days after the second
immunising infection (day 24). Mice in group 1 were given 1.0
ml of a ^{1}/100 dilution of NIMP - R10 ascitic fluid ip. on days
23, 24 and 25 to deplete the neutrophils. Mice in group 2 were
given 1.0 ml of a ^{1}/25 dilution of NIMP - R6 ascitic fluid ip.
on the same days to remove eosinophils. Mice in group 3 received
a combination of 0.5 ml of NIMP - R10 and 0.5 ml of NIMP - R6
ip. at the same concentration as above, and at the same time
points. Mice in control groups 4 and 5 consisted of secondary
immunised mice, or naive mice challenged with 200 L3 respectively.
Resistance to the challenge infection was assessed by counting
cysts 6 days later.

The data in Table 7.5 shows that depletion of neutrophils
or eosinophils, or of both cell types had no effect on the
resistance displayed by mice, which had received two immunising
infections of N. dubius . Since levels of circulating eosinophils,
and neutrophils were decreased (54% decrease in eosinophils, and
a 50% decrease in neutrophil numbers, compared with controls)
in these mice at the time of challenge, it suggests that other
factors or cells must be involved in resistance, possibly

TABLE 7.5

Suppression of immunity to <u>N. dubius</u> in secondary immunised
mice treated with NIMP-R10 and NIMP-R6 ascitic fluid

Group	Status of mice at challenge	mean cyst counts $\pm$ SD
1	Immnue[a] treated with :	
	NIMP-R10	21 ± 5
2	NIMP-R6	20 ± 12
3	NIMP-R10 + NIMP-R6	14 ± 7
4	Immune[a]	20 ± 14
5	Normal	153 ± 22

(a) Micewere made resistant to re- infection by injecting two
     doses of 200 L3 iv. one on day - 24 and a second on day - 10
These mice, together with uninfected controls, were each
challenged on day 0. NIMP-R10, NIMP-R6 or NIMP-R10 + NIMP-R6
were given as indicated in the test. The mice were killed on
day 6 to count the number of encysted larvae.
Group 5 vs. group 4 $P < 0.05$; Group 4 vs. groups 1, 2 and 3
$P > 0.05$ not significant. ('U' test of Mann and Whitney)

activated macrophages at this time.

7.6   REDUCED IN-VITRO LARVICIDAL ACTIVITY OF NEUTROPHILS RECOVERED FROM MICE TREATED WITH NIMP - R10

To confirm that treatment of mice with NIMP - R10 antibody did deplete functionally active neutrophils, from the peripheral circulation, neutrophils were purified from the peripheral blood of mice, which had been treated with NIMP - R10, and tested in-vitro for their ability to impair the infectivity of L3 in the presence of immune serum.

Neutrophils were purified from the blood of mice, either 10 days after a primary immunising infection of 200 L3 iv., or 10 days (day 24) after a second immunising infection of 200 L3 given 14 days after the primary. In addition, neutrophils were purified from the blood of mice, which had received either 1.0 ml of a ^{1}/100 dilution of NIMP - R10 ascitic fluid on days 8 and 9 after primary infection, or days 22 and 23 after a secondary immunising infection of 200 L3, cells were purified on day 10 or 24 respectively. Neutrophils from both the NIMP - R10 treated and untreated primary and secondary immunised mice, were tested for their in-vitro larvicidal activity against exsheathed L3 in the presence of immune serum from N. dubius infected mice by the method outlined in Chapter 5. Ten groups of 5 tubes were set up containing 200 exsheathed L3 in 0.2 ml of supplemented medium. To tubes in 9 of the groups, 0.05 ml of immune serum were added and the tubes incubated for 90 minutes at 37°C. The remaining set of tubes contained L3 in medium alone. After incubation 2 groups of tubes, received 2×10^4 neutrophils from either NIMP - R10 treated or untreated primary infected mice, while a further two groups received neutrophils (2×10^4) from NIMP - R10 treated and untreated secondary infected mice. Similarly, a further 4 groups received 1×10^5 neutrophils from NIMP - R10 treated and untreated primary or secondary infected mice. The tubes were

TABLE 7.6

Reduced in-vitro larvicidal activity of peripheral blood
neutrophils isolated from infected mice which had been treated
with NIMP-R10 ascitic fluid

Incubated larvae	Neutrophils per tube	
in presence of:	2×10^4	1×10^5
Neutrophils from :	mean cyst count $\pm$ SD	
Primary infeced mice(day10)		
Untreated	(3) 83 $\pm$ 12	(4) 78 $\pm$ 10
NIMP-R10 treated	(5) 110 $\pm$ 19	(6) 120 $\pm$ 10
Secondary infected mice(day24)		
Unteated	(7) 76 $\pm$ 22	(8) 69 $\pm$ 16
NIMP-R10 treated	(9) 112 $\pm$ 13	(10) 107 $\pm$ 17
Controls	(1) 131 $\pm$ 9 (no IMS)	
	(2) 136 $\pm$ 8 (+ IMS)	

Figures in parenthesis represent group numbers

(day) represents the day mice were bled for neutrophils

Groups 3, 4,7 and 8 vs. group 1 or 2 P$\leqslant$0.05

Groups 5, 6,9 and 10 vs. groups 1 or 2 P$\geqslant$0.05

Groups 3 vs. 5, 4 vs. 6, 7 vs. 9 and 8 vs. 10 P$\geqslant$0.05

The number of neutrophils recovered from 9 ml of blood was;

secondary infected mice (untreated), 2.4×10^7 cells; day10 suppressed

mice, 6.5×10^5 cells, day 24 suppressed mice, 7.5×10^5 cells.

incubated for a further 48 hours, after which time the contents were fed to mice and cyst counted in the intestinal wall 6 days later.

The data in Table 7.6 indicates, that neutrophils from untreated mice, either 10 days after a primary or a secondary immunising infection of 200 L3, significantly reduced the infectivity of the L3 after incubation, with each of the cell doses tested. Immune serum alone, had no effect on the infectivity of the larvae. The larvicidal activity of neutrophils collected from primary or secondary immunised mice treated with NIMP - R10 were significantly less than that of neutrophils from unsuppressed mice. The cyst counts obtained using neutrophils from NIMP - R10 treated mice, were not significantly different from the controls.

7.7 IN-VITRO LARVICIDAL ACTIVITY OF NEUTROPHILS AND MACROPHAGES FROM N. dubius INFECTED MICE TREATED WITH NIMP - R10

The in-vitro larvicidal activity of cells from peritoneal washouts and peripheral blood neutrophils from NIMP - R10 treated, N. dubius infected mice, was assessed to determine whether another cell type, other than the neutrophil or eosinophil was involved, in the resistance seen in these mice given NIMP - R10, ten days after a primary or a secondary immunising infection of N. dubius.

Two groups of mice were immunised on day 0 with 200 L3 iv. One group of mice received 1.0 ml of a ^{1}/100 dilution of NIMP - R10 ascitic fluid ip. on days 8 and 9 after infection. The remaining group of mice were given 1.0 ml of saline ip. at the same time. A further group of mice were immunised with 200 L3 iv. on day 0 and again on day 14. Half the mice in this group received 1.0 ml of a ^{1}/100 dilution of NIMP - R10 ascitic fluid ip. on days 22 and 23 (8 and 9 days after the second immunising

infection), while the other half received 1.0 ml of saline
ip. at the same time. Peripheral blood neutrophils were
collected from the above groups of mice, either 10 days after
the primary or the secondary immunising infection of 200 L3.
The experiment was designed such that neutrophils were collected
from primary or secondary immunised mice on the same day. All
cells were washed in supplemented medium and resuspended in the
same, to a concentration of 5×10^5 cells/ml. An in-vitro
larvicidal assay was carried out using these cells.

To 8 groups of 5 tubes containing 200 exsheathed L3 in
0.2 ml of supplemented medium, were added 0.05 ml of immune
serum. Tubes in a further 2 groups ie: control groups 1 and 2
respectively, contained exsheathed L3, in either medium alone,
or in medium along with 0.05 ml of immune serum in the absence
of cells. After incubation for 90 minutes at 37°C, 1×10^5 cells
were added to tubes in the 8 experimental groups. Tubes in
groups 3 and 4 received neutrophils from untreated primary
infected mice, while tubes in groups 5 and 6 received neutrophils
from similar mice treated with NIMP - R10. Tubes in groups 7
and 8 received neutrophils from untreated secondary infected
mice, while to tubes in groups 9 and 10 were added neutrophils
from NIMP - R10 treated secondary infected mice. After incub-
ation for a further 48 hours, the contents of the tubes were fed
to mice and cysts counted in the intestinal wall 6 days later.

The data in Table 7.7 indicates, that cells from mice 10
days after a primary or secondary infection with N. dubius were
highly efficient at impairing the infectivity of L3 in-vitro.
Furthermore, NIMP - R10 treatment of these mice had no affect
on the activity of macrophages in-vitro. Neutrophils from mice
taken 10 days after a primary or a secondary infection of
N. dubius, were also active against the larvae in-vitro, however
treatment with NIMP - R10 resulted in a population of neutrophils,

TABLE 7.7

In-vitro larvicidal activity of neutrophils and peritoneal cells
from N. dubius infected mice treated with NIMP-R10 ascitic fluid

Larvae incubated with:	Treatment of donor mice (days injected)	Cells tested	
		Peritoneal cells	Neutrophils
		mean cys count $\pm$ SD	
Cells from primary infected mice collected on day 10	None	(3) 75 $\pm$ 6	(4) 107 $\pm$ 7
	NIMP-R10(d8,9)	(5) 83 $\pm$ 10	(6) 162 $\pm$ 19
Cells from secondary infected mice collected on day 24	None	(7) 94 $\pm$ 11	(8) 103 $\pm$ 15
	NIMP-R10(d22,23)	(9) 95 $\pm$ 11	(10) 144 $\pm$ 14
Controls			
L3 in medium alone	(1) 166 $\pm$ 7		
L3 in medium + IMS	(2) 164 $\pm$ 8		

Figures in parenthesis represent group numbers

(d 8,9)and(d22,23) days when mice treated with NIMP-R10

Groups 3,4,5,7,8 and9 vs. groups 1 or 2 $P < 0.05$

Group 2 vs. groups 6 and 10 $P > 0.05$ not significant

Group 3 vs. 5, 7 vs.9 $P > 0.05$ not significant

Group 4 vs.6 and 8 vs. 10 $P < 0.05$

which were inactive against L3 in-vitro. The data suggests that the resistance seen in NIMP - R10 treated mice, may be due to another cell type, other than the neutrophil, possibly activated macrophages. Furthermore, NIMP - R10 treatment of mice may preferentially deplete the proportion of neutrophils with larvicidal activity.

7.8   CONCLUSIONS

The effect of neutrophils and eosinophils in the resistance of mice to reinfection with N. dubius, was investigated by treatment of immunised mice with monoclonal rat antibodies against granulocytes at the time of rechallenge. NIMP - R10 treatment of mice resulted in a significant fall in the number of circulating neutrophils in mice, during the first 2-3 days of treatment ie: at the time of rechallenge. NIMP - R10 treatment appeared to be specific, in that no decrease in macrophage, lymphocyte or eosinophil numbers resulted. When mice were challenged 5 days after a primary infection, treatment with NIMP - R10 completely suppressed immunity, even after passive transfer of immune mouse serum. In contrast, the immunity of mice rechallenged 10 days after a primary infection, was only partially suppressed by NIMP - R10, while immunity of secondary immunised mice, which had been rechallenged 10 days after a second immunising infection of N. dubius was unaffected.

Neutrophils appear to play a predominant, if not exclusive role in the early resistance of mice to reinfection with N. dubius larvae, but they become less important with time, such that, after two immunising infections, other cell types are equally as important in conferring resistance to reinfection.

Secondary immunised mice exhibit a pronounced eosinophilia , and it has been speculated that eosinophils, may play an active role in resistance at this stage of infection. However, treatment of mice with an anti-eosinophil monoclonal antibody

NIMP - R6 caused no significant loss of immunity to reinfection, although a pronounced reduction in eosinophil levels did occur. This will be discussed later.

Treatment of mice with anti-neutrophil serum NIMP - R10 did not completely deplete all circulating neutrophils, however the remaining cell population when purified from these suppressed mice, was shown to be inactive against larvae in-vitro when compared with neutrophils from unsuppressed mice. 'Stimulated' neutrophils may be preferentially depleted from mice by NIMP - R10 antibody, leaving a less active neutrophil population unable to damage larvae in-vitro. In contrast the the early stages of infection, where neutrophils appear to be the main effector cells, in the later stages of infection activated macrophages may be important effector cells, since peritoneal cells collected from mice 10 days after a primary or a secondary infection were highly efficient in damaging larvae in-vitro. Depletion of neutrophils from these mice had only partial, or no effect on resistance to reinfection.

CHAPTER 8

DISCUSSION

Of the various types of cell considered to be potential
effectors against helminth parasites in mamalian hosts, the
eosinophil and neutrophil have recently attracted considerable
attention (Ogilvie, 1980; Mackenzie et al., 1981; McLaren et al.,
1981). Each of these cell types have been shown to be capable
in-vitro of damaging Schistosomula of S. mansoni, or newborn
and infective larvae of T. spiralis (McLaren et al., 1977);
Incani and McLaren, 1983). These findings, together with the
pronounced eosinophilia characterising most helminth infections
have raised the possibility that these cells play an active role
in immunity.

N. dubius infections in mice, provide a useful laboratory
model for the study of effector cells in immunity and their
interaction with a helminth parasite. Damage to, and loss of
infectivity of larvae can be assessed directly and simply after
challenging mice, by counting encysted larvae within the intest-
inal wall 5-6 days later, or by counting adult worms in the
intestine at 14 days. In addition, effector cells such as,
eosinophils and neutrophils can be purified relatively simply
from the peripheral blood of mice, as outlined in Chapter 3.

Histopathological studies of the small intestine of mice
infected with N. dubius, have shown that macrophages, neutrophils
and eosinophils, may surround encysted larvae in the muscularis
mucosa of mice immune to reinfection (Jones, 1974; Jones and
Rubin, 1974). Previous in-vitro studies have shown, that
macrophages from mice infected with N. dubius, in an activated
state, can damage and kill L3 (Chaicumpa and Jenkin, 1978).

Eosinophils and neutrophils purified by density gradient
centrifugation from the peripheral blood of mice, resistant to
reinfection with N. dubius were assessed, by light microscopy
for their ability to adhere to the different parasitic stages

of N. dubius, as outlined in Chapter 4. It was observed in the present study, that eosinophils which adhere to N. dubius became flattened against the parasite surface, in contrast to neutrophils, which were seen to flatten only slightly. This is in accord with similar observations made during the interaction of human or rat eosinophils and neutrophils with the surface of S. mansoni (Vadas et al., 1980) and T. spiralis and N. brasiliensis (Mackenzie et al., 1981). The adherence of polymorphonuclear cells to larvae, was studied after the parasite had been sensitised with either N. dubius immune serum or normal serum, in the presence of Ca^{2+} and Mg^{2+} ions, EDTA or EGTA.

In the work presented, the use of VB^{2+}, VB^{2+} - EGTA or VB^{2+} - EDTA diluents enabled the efficacy of opsonisation with antibody or complement to be evaluated in terms of their ability to mediate cell adherence in-vitro.

All nematodes during their life cycle, undergo a series of moults in which the surface cuticle is shed. It has been shown for T. spiralis, that the surface cuticle expresses molecules which change qualitatively after moulting or quantitatively during growth of the worms (Philipp et al., 1980, 1981; Jungery, Clark and Parkhouse, 1983). Furthermore, it was shown that rats infected with T. spiralis mount a stage-specific antibody response the the parasite surface. The time course of appearance of antibody to the surface antigens mirrored the time course of appearance of antibody that mediated eosinophil adherence to the surface of each stage. These stage specific antigens, in the case of T. spiralis may be the targets for eosinophil mediated destruction of the parasite.

In the interaction of eosinophils and neutrophils with the infective third-stage sheathed larvae of N. dubius, it was observed that maximal adherence occurred only on larvae which had

been incubated under conditions, which allowed complement activation. N. dubius has the ability to activate the alternative pathway of complement (Prowse et al., 1979) and these results, suggested that the larvae were sensitised with C3 molecules and that the cells were bound to the larvae via their C3 receptors (Lopez et al., 1981). The additional involvement of antibody was evident from the binding of cells to larvae treated with immune serum - EDTA (in which no complement activation can occur), since no cells bound to larvae sensitised with normal serum - EDTA.

The adherence of eosinophils and neutrophils to the exsheathed infective third-stage larvae showed a different pattern from that observed with the sheathed form. The number of cells binding to the surface of exsheathed L3 treated with immune serum in VB^{2+} or VB^{2+} - EDTA or with normal serum in VB^{2+} were similar. Thus, adherence of eosinophils or neutrophils to the exsheathed larvae occurred via specific antibody, as well as by complement. Since few cells bound via specific antibody to the sheathed larval form, it appears that the sheath possesses fewer antigenic sites than the surface of the exsheathed L3. However, differences in antigenic content can only be rigourously examined by measuring antibody binding directly, rather than via secondary phenomena such as cell adherence, which depends on many factors, including the type of antibody bound.

Adhesion of both neutrophils and eosinophils to 48 hour and 72 hour post-infective larvae, which represent growth phases of the exsheathed infective L3 larvae, occurred equally well via Fc receptor or C3 receptor interactions. However, the adherence of the cells to 96 hour post-infective larvae was greatly reduced, particularly so, when one takes into account the larger size of these worms, when the larvae were sensitised with immune serum in VB^{2+} - EDTA rather than in VB^{2+}, suggesting that a change

occurs in the surface properties of these larvae, following
the moult into the 4th stage. Cell adhesion to the adult worms
was similarly reduced and seemed to be mediated mainly by
complement. It appears from these results that the cuticle of
the 4th and 5th stages of N. dubius, might carry fewer exposed
antigenic sites and fewer complement-fixing sites, than that of
the preceding stages. Cell binding occurred mainly around the
head and tail regions, indicating that the antibodies promoting
adhesion, might be directed against secretory/excretory antigens.

In accord with similar observations made by Lopez et al.,
(1981), eosinophils and neutrophils from N. dubius infected mice
were found to bind to and ingest erythrocytes sensitised with
IgG_1, IgG_{2a}, IgG_{2b} but not IgM anti-sheep red blood cell immumo-
globulin isotypes. However, when adherence of these polymorpho-
nuclear cells to the surface of N. dubius was assessed, the cells
were found to adhere to exsheathed L3 only in the presence of
IgG_1 (but not IgG_{2a} or IgG_{2b}) immunoglobulin purified from
N. dubius immune serum. This would suggest an absence of suffic-
ient levels of specific antibody in the latter two isotypes.
Titration of the ability of an IgG fraction from immune serum,
which was predominately IgG_1 to promote adherence of eosinophils
and neutrophils to the surface of the different stages in the
life cycle of N. dubius, suggested an increase in the number of
determinants on the parasite surface, against which the IgG was
directed, as the parasite matured during it's life cycle, from
the exsheathed L3 stage, to the 48 hour, 72 hour post-infective
larval stages.

In conclusion, the data indicates that the adherence of
eosinophils and neutrophils to N. dubius in-vitro can be mediated
both by parasite-bound antibody and by parasite-bound complement,
presumably C3 and it's fragments. C3 has in fact, been detected
by immunofluorescence on the surface of serum-sensitised

N. dubius larvae (Ey, Personal Communication). In addition, it appears that the antigenic properties of the cuticle of the parasite change during the life cycle. The antibodies which are present in the serum of infected mice, and which promote eosinophil or neutrophil adherence, are primarily directed to the stages of N. dubius that penetrate and dwell within the intestinal mucosa. Very little specific antibody promoting cell adherence is directed against the sheath of the infective third-stage larval form, or to the adult worm, which dwells in the lumen of the intestine. Later experiments examined the potential of eosinophils and neutrophils as effector cells in immunity to N. dubius (Chapter 5). The effect of cells on larval infectivity was assessed after incubation of polymorphonuclear cells with L3 in-vitro. Peripheral blood eosinophils and neutrophils, were purified from N. dubius resistant mice, resulting in cell preparations comprising 94% - 98% neutrophils, or 63% eosinophils. Both the neutrophils and eosinophil enriched cell population exhibited larvicidal activity in the presence of fresh immune serum. The activity of the eosinophil-enriched preparation was attributed to it's eosinophil content, since lymphocytes and monocytes (the predominant contaminants) were themselves devoid of activity and because within this cell population, eosinophils constituted the predominant cell type which adhered to the larvae. On a cell for cell basis, neutrophils from infected mice, consistently exhibited a larvicidal activity at least equal to and usually greater than that of the eosinophils. Moreover, the neutrophils were only marginally more active in fresh IMS than in fresh normal serum, indicating that larvae sensitised with complement alone, were damaged almost as efficiently as those sensitised with antibody plus complement. In contrast, the eosinophil preparation appeared to damage larvae, only in the presence of both antibody and complement. When only specific

antibody was present, ie: using heated or methylamine-
inactivated immune serum, or an immunoglobulin fraction from
immune serum, neither cell preparation exhibited any larvicidal
activity, demonstrating that the presence of complement was
necessary for the activity of both cell types in this system.
It is questionable, however, whether the level of opsonic
complement in the interstitial fluid surrounding the developing
larvae in the muscularis mucosa of the small intestine, is as
high as that of serum. It seems reasonable to expect that any
larvicidal attack by neutrophils in-vivo, would require the
presence of antibodies, as well as complement, even though serum
complement can be activated in-vitro via the alternative pathway
by all stages of the parasite (Prowse, Ey and Jenkin, 1979a).

Similar results with other helminths suggest that opsoni-
zation with C3 through the activation of complement by parasite-
bound antibodies, or by the parasite itself, may be of consider-
able importance in-vivo. For example, complement has been found
to facilitate in-vitro antibody-dependent cell-mediated damage
to schistosomula of S. mansoni (Anwar et al., 1979, Incani and
McLaren, 1983), both newborn and infective larvae of T. spiralis
(Mackenzie et al., 1980) and microfilariae of Onchocerca
volvulus (Green, Taylor and Aikawa, 1981). Schistosomula (Anwar
et al., 1979) and the infective larvae of N. brasiliensis
(Mackenzie et al., 1980) have been reported to be damaged in-vitro
by effector cells in the presence of complement alone.

Although neutrophils are mentioned in many instances in
the literature as being less effective than eosinophils against
helminths in-vitro, they are, none the less, clearly able to
effectively damage a variety of parasites. Vadas et al., (1980b)
found that human neutrophils were highly active in an assay
involving the release of ^{51}Cr from labelled schistosomula,
although they did not appear to inflict microscopically-

detectable damage on the parasite. However, neutrophils can cause lesions in the tegument of S. mansoni, which are detectable by electron microscopy (Incani and McLaren, 1983). Neutrophils have also been observed to penetrate the cuticle and extend pseudopodia into the underlying tissues of second-stage larvae of Ascaris suum (Thompson et al., 1977). These cells are further reported to be active in-vitro as effector cells against microfilariae of O. volvulus (Greene et al., 1981) and D. viteae, against which they were apparently the major effector cell type (Rudin et al., 1980). Mehta et al., (1981) reported that both neutrophils and eosinophils adhered to W. bancrofti larvae in the presence of IgG antibody, but only neutrophil adhesion resulted in marked toxicity to the parasite. Even in the case of T. spiralis, in which eosinophils are more more active on a cell for cell basis, neutrophils exhibited potent larvicidal activity (Kazura and Aikawa, 1980). It has also been reported that myeloperoxidase from neutrophils, was more efficient than eosinophil peroxidase in the in-vitro killing of T. spiralis newborn and infective larvae (Buys, Wever and Ruitenberg, 1984). The results reported here on the effect of neutrophils on N. dubius, thus have analogies in other systems.

Possibly the most interesting and important finding of this study, is that neutrophils from normal mice, were unable to damage N. dubius L3 in-vitro even in the presence of antibody and complement, conditions under which neutrophils from immune mice were highly effective. It seems therefore, that neutrophils from mice infected with N. dubius are altered in their activity in some way. This is the first time, to our knowledge, that the killing in-vitro of a metazoan parasite by neutrophils has been shown to require these cells to be altered or 'stimulated'. Whether the alteration of neutrophil activity requires the participation of accessory cells, as does the activation of

macrophages, is at present not known. Veith and Butterworth, (1983), have compared mononuclear cell culture supernatants from patients with an ongoing eosinophilia and normal patients for their ability to enhance eosinophil killing of Schistosomula of S. mansoni. Both culture supernatants enhanced the killing activity of eosinophils in the presence of immune serum.

The appearance of neutrophils with larvicidal properties, is not a specific response to infection with N. dubius, however, since they also appear in the peripheral blood of mice, which have been infected with S. enteritidis 11RX. In addition, killed vaccines, such as frozen - thawed L3, or adult worm extracts when injected into mice, also resulted in the appearance of neutrophils with in-vitro larvicidal activity. Unfortunately, these types of vaccines fail to protect mice against infection in-vivo, which suggests that the antibody response in these mice may not be protective.

Altered neutrophils, are present in the peripheral blood 4 days after a primary N. dubius infection, and appear to be present up to at least 6 weeks after a secondary infection. It is interesting to note, that eosinophils from patients with schistosomiasis have been found to be significantly better, than those from normal individuals in causing antibody-dependent ^{51}CR release from labelled schistosomula, whilst neutrophils from both sources, exhibited no difference in activity (Vadas et al., 1980b).

There is a substantial literature, indicating that the anti-microbial and cytotoxic properties of neutrophils can be enhanced under certain conditions. For example, human neutrophils are reported to acquire an enhanced capacity to phagocytose and kill Serratia marcescens after exposure to supernatants of activated human mononuclear cells (Cross and Lowell, 1978) and exposure to platelet activating factor or endotoxin can stimulate a variety of functions, including exocytosis, respiratory burst

activity and adhesiveness (Shaw et al., 1981, Dahinden, Galanos and Fehr, 1983). Moreover, the in-vitro antibody-dependent tumouricidal activity of human neutrophils and eosinophils has been found to be rapidly enhanced by granuloycte-specific-colony-stimulating factors (CSF) (Vadas, Nicola and Metcalf, 1983). These findings indicate, that the functional capabilities of 'normal' neutrophils can be changed, as a consequence of infection or inflammation. Presumably factors, such as the CSFs regulate these changes, and it would be of interest to determine whether or not 'normal' mouse neutrophils exposed to mouse neutrophil-specific CSF acquire the capacity to damage N. dubius L3.

One clearly needs to be cautious in interpreting data from different studies, in which the activity of neutrophils obtained from either uninfected, or infected individuals, has been reported. Although 'normal' neutrophils are apparently active in other in-vitro helminth killing systems (Kazura and Aikawa, 1980, Greene et al., 1981, Mehta et al., 1981), the use of neutrophils from uninfected individuals, may in part account for the poor activity of these cells in some in-vitro assays, particularly if the cells are tested in the absence of complement (Dessein et al., 1983) and the antibodies present, are of an isotype, which bind poorly to the cell-surface Fc receptors.

In summary, the present study implicates, both the neutrophil and the eosinophil in immunity to N. dubius. From the work presented in this thesis, it is not clear whether the neutrophils are in an 'activated' state, similar to that reported for macrophages. The increased larvicidal activity of neutrophils may represent a shift in the neutrophil population, following infection such that they are in a more mature state.

It was found in the present study (Chapter 6), that when mice infected with N. dubius, only 4 days previously were

injected with serum or purified immunoglobulins from immune mice, they were partially resistant to a challenge dose of larvae administered at the same time as the serum. The administration of immune serum to normal mice induced no resistance to a challenge infection. On the basis of these results, it was considered that 4-day-infected mice, might already possess a population of cells capable in the presence of antibody of preventing larval development. The requirement for serum antibodies is almost certainly attributable to the limiting amount of naturally-acquired antibody available at this time. The presence of 'stimulated' neutrophils in these mice, reported (Chapter 5) was confirmed, using purified peripheral blood neutrophils in the in-vitro larvicidal assay. Peritoneal washout cells from the same mice were inactive in this assay, however, indicating that activated macrophages were not yet present. Thus, it seemed possible that the partial immunity conferred on 4-day-infected mice by the transfer of immune serum or immunoglobulins, was due to the action of 'stimulated' neutrophils on the invading larvae. The capacity of such neutrophils to protect mice against infection by N. dubius was unambiguously demonstrated by the transfer of immunity to naive mice, by a combination of purified neutrophils and immune serum, but not by cells or serum alone.

These findings further demonstrate that 'normal' neutrophils ie: those present in uninfected mice, are incapable of providing any detectable degree of resistance against infection by N. dubius, even in mice, which have been injected with immune serum. However, such mice exhibit significant resistance to infection, if they are additionally injected with neutrophils from infected mice. There seems to be a necessity for neutrophils to be 'altered' (eg: by infection of the donor or test mouse) before they are capable of damaging N. dubius L3 either

in-vitro in the presence of antibody and complement, or in-vivo.

Previous attempts to transfer immunity against N. dubius have had mixed success. A number of investigators, found that normal mice injected with small volumes ($\leqslant 1$ ml) of serum had no significant resistance to a challenge infection (Panter, 1969[b]; Cypess, 1970; Chaicumpa et al., 1976). More recently, it has been reported, that the infectivity and survival of N. dubius is severely impaired in female NIH mice treated with multiple doses of immune serum (Behnke and Parish, 1979). Immunity was significantly enhanced in animals given both immune serum and mesenteric lymph node cells from immune mice, particularly when sub-lethally irradiated larvae were used for the challenge dose (Behnke and Parish, 1981). It is possible that NIH mice, which are a strong-responder strain (Williams and Behnke, 1983) are unusual in this respect, perhaps possessing a pool of activated cells, which may mediate immunity in the presence of antibody. Whatever the reason for these differences, one clearly needs to be cautious in analysing the mechanisms of transferred immunity. In addition, to its' content of antibodies, immune serum may contain sufficient amounts of lymphokines and granulocyte colony-stimulating factors (Vadas, Nicola and Metcalf, 1983) to activate effector cells in the recipient mice, particularly if appreciable volumes of serum are transferred.

Similar restrictions apply to the data from cell transfer studies. Immunity to N. dubius has been passively transferred to normal mice with spleen cells (Cypess, 1970) and mesenteric lymph node cells (MLNC) (Behnke and Parish, 1981) from immune mice. The transfer of these heterogeneous cell populations, perhaps also with small quantities of cell associated parasite antigens, may be expected in the recipient mice to result in lymphokine release, activation of the mononuclear phagocytic system and secretion of parasite specific antibodies. Indeed,

Mice given immune MLNC have been found to have elevated IgG_1 levels (Pritchard et al., 1983).

The transfer of immunity by purified neutrophils, together with immune serum, which alone gives no immunity, is less likely to involve interactions in the recipient induced by lymphocytes, or monocytes as the level of these cells in the transferred cell population was very low (approximately $2 \pm 1\%$). Furthermore, lymphocytes/monocytes isolated from the same mice from which the neutrophils were obtained were inactive against L3 in-vitro (Chapter 5). The transfer of small numbers of contaminant lymphocytes or monocytes, present in the neutrophil preparations, is unlikely to have had any direct effect on larvae in the challenge dose. Although one cannot exclude the possibility that these cells release factors, which activated effector cells, other than neutrophils in the recipient mice, this seems unlikely, especially in view of the number of immune MLNC needed to achieve adoptive immunity in NIH mice given immune serum ($> 10^6$ cells/mouse; Behnke and Parish, 1981). One is therefore, left with the conclusion that the transferred neutrophils were directly responsible for the immunity exhibited by the mice.

There was a clear requirement for antibody by the neutrophils, both IgG and IgM fractions of immune serum being able to induce immunity in 4-day-infected mice. Murine neutrophils possess receptors for IgG and C3-coated erythrocytes, but not IgM-coated cells (Lopez, Strath and Sanderson, 1981). The resistance transferred via the IgM fraction, may have been due to the activation of complement by parasite-bound IgM antibodies. This could promote the adhesion of neutrophils via their C3 receptors to the larval surface, as has been demonstrated to occur in-vitro.

In summary, from the data in Chapter 6, it appears that neutrophils are capable of directly damaging N. dubius larvae

both in-vitro and in-vivo in the presence of antibody and complement. Not only can immunity be transferred by purified neutrophils from immune mice, but it appears likely, that neutrophils are responsible for the protection observed in 4-day-infected mice given immune serum. The importance of neutrophils compared with macrophages and eosinophils in natural immunity to this parasite, remains to be determined however.

The role of eosinophils and neutrophils in the acquired resistance of mice to reinfection by N. dubius was further assessed in-vivo (Chapter 7). This was achieved by injecting previously infected mice with monoclonal antibodies specific for murine polymorphonuclear cells (NIMP - R10 and NIMP - R6), at the time of administering a challenge dose of infective larvae.

Treatment of both primary and secondary infected mice with NIMP - R10 caused a significant fall in the number of neutrophils in the blood or peritoneal cavity during the ensuing 2-5 days, while no major changes were observed in the number of macrophages or eosinophils. Treatment of secondary immunised mice with NIMP - R6 caused a significant fall in eosinophil numbers without a significant effect on other cell types.

Although an eosinophilia in mice, is a characteristic of infection by N. dubius, and full resistance was reported to correlate with the appearance of eosinophils in infected mice (Prowse et al., 1978a), these cells in-vivo in a cell transfer experiment were ineffective in conferring resistance on naive mice (Chapter 6). Furthermore, depletion of eosinophils from the circulation of mice 8-10 days after a secondary challenge infection by 200 L3, by injection of NIMP - R6 ascitic fluid had no effect on the resistance of these mice to reinfection. The mice remained fully protected against the challenge infection, even though eosinophil levels were significantly reduced. The

role of eosinophils in immunity to N. dubius remains difficult to resolve from the above data, even though these cells were larvicidal in-vitro. Although there was a decrease in the levels of eosinophils after NIMP - R10 treatment of mice, the question remains as to whether the eosinophil were involved in damage to the larvae, the decrease in eosinophil numbers were insufficient to abolish the resistance of the mice. It was not possible to test the residual population of eosinophils in these mice, for their larvicidal activity as had been done, for neutrophils since there were insufficient numbers in the circulation to make this experiment feasible. In contrast, depletion of neutrophils from N. dubius infected mice by treatment with NIMP - R10 ascitic fluid given 4 days after a primary infection of 200 L3 (which is known to induce 'stimulated' neutrophils), resulted in a significant fall in neutrophil numbers, along with the total loss of resistance to the challenge infection, despite the fact that, these mice also received immune serum passively. However, depletion of neutrophils by NIMP - R10 treatment, 10 days after a primary infection of 200 L3 resulted in only a partial loss (50%) of resistance to challenge by N. dubius L3, given on day 10 after the primary immunising infection. Depletion of eosinophils or neutrophils, from mice 8-10 days after a secondary infection, had no effect on their resistance to a subsequent challenge.

The observations in Chapter 7, indicate that in the early stages of infection by N. dubius neutrophils, providing immune serum is passively transferred, are able to confer a degree of resistance on the host. As the infection proceeds, macrophages became 'activated', such that at day 10, after a primary infection, these cells were larvicidal in-vitro. Neutrophils may therefore, at this stage, act in concert with macrophages in conferring resistance to mice, since NIMP - R10 depletion of

neutrophils only partially abolished immunity and furthermore, eosinophils are not present in the circulation or peritoneal cavity at this time. After a secondary immunising infection, macrophages, neutrophils and possibly eosinophils, together with specific antibody, may act together to confer full protect- ion on mice. Neutrophil or eosinophil depletion at this time, would therefore be compensated for by macrophages, which have larvicidal activity. Unfortunately, an anti-macrophage serum was not available to determine what effect depletion of macro- phages from mice would have had on resistance.

In the in-vitro studies, neutrophils from mice treated with NIMP - R10 were found to be inactive against larvae in the presence of immune serum, when compared to neutrophils from untreated mice. Macrophages activity against L3 in-vitro was not affected by NIMP - R10 treatment of mice, from which the cells were collected.

The inactivity of neutrophils from NIMP - R10 treated mice suggested that this monoclonal antibody depleted the population of neutrophils with larvicidal activity. However, as mentioned previously, it remains unclear in the present study, whether neutrophils with increased larvicidal activity represent an 'activated' cell population, or a shift to a more mature cell population as a consequence of infection.

In summary from the in-vitro studies, three phagocytic cell types appear to play a role in immunity to N. dubius. Previous investigators have implicated 'activated' macrophages (Chaicumpa and Jenkin, 1978), and the present study has implicated both eosinophils and neutrophils. Furthermore, mice can be partially protected against infection by passive transfer of 'stimulated' neutrophils in conjunction with immune serum, but not by the passive transfer of eosinophils, suggesting a role for neutro- phils as effector cells in-vivo as well as in-vitro.

The neutrophil appeared to act in the early stages of a primary infection. Other cell types became equally important in resistance, as the infection proceeds. Eosinophils on the other hand, appeared not to be efficient effector cells in immunity to N. dubius in the in-vivo experiments, which contrasted with the findings from the in-vitro situation. From the data the role of the eosinophil in immunity to N. dubius remains unclear and further work is required in this area.

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