THE IMMUNE RESPONSES OF *PERIPLANETA AMERICANA* TO *HYMENOLEPIS DIMINUTA* AND *MONILIFORMIS MONILIFORMIS*

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A thesis presented for the degree of Doctor of Philosophy in the University of Glasgow, Faculty of Science, Departments of Zoology and of Cell Biology.

Declaration:

I declare that the work presented in this thesis is original and that the research and composition of the text was carried out by me.

MARGARET MARY CARR

August 1990

IF

If you can keep your head when all about you
  Are losing theirs and blaming it on you;
If you can trust yourself when all men doubt you,
  But make allowance for their doubting too;
If you can wait and not be tired by waiting,
  Or being lied about, don't deal in lies,
Or being hated don't give way to hating,
  And yet don't look too good, nor talk too wise.

If you can dream - and not make dreams your master;
If you can think - and not make thoughts your aim;
If you can meet with Triumph and Disaster
  And treat those two imposters just the same;
If you can bear to hear the truth you've spoken
  Twisted by knaves to make a trap for fools,
Or watch the things you gave your life to broken,
  And stoop and build 'em up with worn-out tools.

If you can make one heap of all your winnings
  And risk it on one turn of pitch-and-toss,
And lose, and start again at your beginnings
  And never breathe a word about your loss;
If you can force your heart and nerve and sinew
  To serve your turn long after they are gone,
And so hold on when there is nothing in you
  Except the Will which says to them: "Hold on!"

If you can talk with crowds and keep your virtue
  Or walk with Kings - nor lose the common touch,
If neither foes nor loving friends can hurt you,
  If all men count with you, but none too much;
If you can fill the unforgiving minute
  With sixty seconds' worth of distance run,
Yours is the Earth and everything that's in it,
  And - which is more - you'll be a Man, my son!

Rudyard Kipling

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This thesis is dedicated to my parents.

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LIST OF ABBREVIATIONS

a	Asialo (prefix)
AcP	Acid phosphatase
BSA	Bovine serum albumin
BSM	Bovine submaxillary mucin
citEDTA	citrate ethylenediaminotetracetic acid (disodium salt)
CMF-EDTA	Calcium/magnesium free EDTA
CO	Coagulocyte
Conc.	Concentrated
D73	cockroach haemocyte maintainance medium
DAB	3,3'-diaminobenzidine-tetra-HCL
DMSO	Dimethyl sulphoxide
DOPA	L-3,4-dihydroxyphenylalanine
FITC	Fluorescein isothiocyanate
FCS	Foetal calf serum
HBS	Hepes buffered saline
Hd	Hymenolepis diminuta
H.I.	Heat inactivated
H_2O_2	Hydrogen peroxide
Imm.	Immature
kD	Kilodalton
LPS	Lipopolysaccharide
Mm	Moniliformis moniliformis
Mat.	Mature
n	Native (prefix)
NaF	Sodium fluoride
PBS	Phosphate buffered saline
Perox	Peroxidase
P.I.	Post-injection
PL	Plasmatocyte
PSM	Porcine stomach mucin
SOD	Superoxide dismutase
THC	Total haemocyte count
Zs	Zymosan A supernatant

THESIS SUMMARY

This thesis examines the insect immune system using the cockroach, Periplaneta americana as a model. Host immunocompetence has been investigated by studying the cellular immune response to infection with Moniliformis moniliformis (Acanthocephala) and Hymenolepis diminuta (Cestoda). Studies of this nature are relevant to medicine, veterinary science and agriculture because of the deprivation and suffering caused by insects as disease vectors and as pests.

A general introduction (Chapter 1) provides information on the life-cycles and biology of the parasites used in this study and gives an overview to the study of invertebrate immunology, emphasising functional aspects of the insect cellular immune response. In Chapter 2 the experimental procedures used throughout the work are detailed.

CHAPTER 3 - TOTAL CELL COUNTS, PHAGOCYTOSIS AND NODULE FORMATION

Chapter 3 investigates the circulating blood cell number, the phagocytic capacity of the haemocytes and the tendency of cells to aggregate (nodule formation) after immune manipulation. Hymenolepis oncospheres significantly increased both circulating cell number and phagocytic capacity of the blood cells. Both Zs, containing β1,3-glucans, and Moniliformis acanthors lowered the cell count but only Zs stimulated nodule formation. The total cell count remained unaltered in animals parasitised with a known dose of Moniliformis acanthellae. These results are discussed in relation to parasite survival and immunocompetence.

It can be concluded that Hymenolepis has a stimulatory effect on the immune system of the cockroach.

CHAPTER 4 - ENCAPSULATION AND SPREADING

Haemocytes in the in vitro encapsulation assay retained the ability to discriminate between surfaces of different charge. Examination of the ultrastructure of haemocytes encapsulating target particles revealed that the plasmatocytes are the main capsule forming cells in vitro. This was further supported by the observation of phagocytic cells incorporated into capsules. Cytochemical processing of capsules showed that acid phosphatase and peroxidase are present in the adherent cells.

The capsule thickness around beads encapsulated in vivo was reduced when animals were Hymenolepis infected and unaltered after Moniliformis infection. These results are discussed in the light of evidence on cell counts from the previous chapter. Analysis of the ability of haemocytes to spread on protein-coated glass after infection indicated that reduced capsule thickness had not resulted from alterations in cell spreading.

CHAPTER 5 - THE FLOW CHAMBER

The discriminatory abilities of the haemocytes were tested in Chapter 5 and correlated with the activities of the lectin-ligand interaction thought to be associated with cellular recognition of "non-self" in invertebrates.

Cockroach haemocytes possess a galactose-specific lectin on their cell membranes and cell adhesion to galactose rich (porcine stomach mucin) and galactose poor (bovine submaxillary mucin) glycoproteins has been investigated. A method for assessing vertebrate blood cell adhesion in vitro under flow conditions was modified for use with

insect haemocytes.

Haemocytes adhered better to the galactose-rich porcine stomach mucin (PSM) compared with the galactose-poor (but sialic acid-rich) bovine submaxillary mucin (BSM). Infection with Hymenolepis improved the adhesion of cells to both BSM and PSM which may indicate a generally enhanced acuity of recognition and immune responsiveness. Zs stimulated improved adhesion to BSM but this was associated with reduced adhesion to PSM and this is likely to reflect subpopulation depletion as haemocytes participate in nodule formation in vivo. Finally, Moniliformis acanthors and acanthellae had no effect on cell adhesiveness in the flow chamber. These results are discussed in relation to immune recognition and parasite survival. Since Hymenolepis and Zs alter adhesion to the glycoproteins whereas Moniliformis does not, it can be concluded that the former are recognised by the immune system whereas the latter is not. Evasion of recognition mechanisms rather than suppression of effectors by Moniliformis is suggested by these data.

CHAPTER 6 - CYTOCHEMISTRY

The intracellular enzymes, acid phosphatase, peroxidase and lysozyme have been localised in the granules of cockroach haemocytes. The plasmatocyte subpopulation is heterogeneous with respect to these enzymes. Approximately 90% of plasmatocytes contain acid phosphatase, 30% contain peroxidase and 40% contain lysozyme. The phagocytic subpopulation of plasmatocytes (10% in the naive animal) do not contain peroxidase. The role of peroxidase in the immune response is discussed in the light of this finding and other circumstantial evidence and it is proposed that the cytochemical assay may be detecting a subtype of

prophenoloxidase, called a laccase.

Haemocytes did not reduce nitroblue tetrazolium after phagocytosis suggesting that the respiratory burst does not occur in these cells and that the peroxidase detected by cytochemical means is not associated with the evolution of toxic oxygen metabolites.

Intracellular acid phosphatase was unaffected by all pre-treatments whereas peroxidase activity was significantly elevated by Hymenolepis infection and unaffected by Zs injection and infection with Moniliformis acanthellae. These results have important implications for understanding immune activation and parasite survival.

CHAPTER 7 - GENERAL DISCUSSION

In this chapter a summary diagram collates the evidence on cell behaviour from preceding chapters and draws conclusions on immune responsiveness and parasitism.

The evidence for immune stimulation by Hymenolepis shows that this parasite is recognised as non-self by the host and the immune system mounts an appropriate cellular response to combat the infection. Although immune suppression by Moniliformis is not excluded, the present work strongly supports immune evasion as the mechanism of survival by this parasite.

The work presented in this thesis provides a fuller understanding of how the cellular immune system of the insect responds to infection and provides a sound basis for further studies on host-parasite interactions using this model system.

CHAPTER 1 - GENERAL INTRODUCTION

CHAPTER 1 - GENERAL INTRODUCTION

This thesis combines the study of cell biology, more specifically immunology, and parasitology to provide information on the immune response of insects to helminithic invasion. The introduction is divided into seven sections. The first section examines the rationale for studying invertebrate immunology. Later sections deal with parasite-host interactions and are followed by a detailed discussion of the components of the invertebrate immune response.

1.1. WHY STUDY INVERTEBRATE IMMUNOLOGY?

Insects destroy crops and transmit many serious diseases to humans and livestock causing at least, reduced fitness, and at worst, death. In the 1960s insecticides were hailed as having revolutionised preventative medicine in the tropics but these hopes were destroyed with the emergence of resistant strains of insect. As public opinion changes on environmental pollution, particularly chemical contamination, biological control of agricultural and medical pests becomes a desirable alternative to insecticides.

For biological control programmes to be successful, it is essential that as much as possible is known of the biology of the pest. Although other control methods exist, such as releasing sterilised males into the gene pool, control by pathogenic organisms and parasites is of most relevance to the present study. One important consideration in control regimes is to understand how the insect defends itself from infection before we attempt to interfere with parasite or host susceptibility. Herein lies the need to study invertebrate immune systems.

1.2. THE BARRIERS TO INFECTION

To gain access to the internal environment of the host, the parasite must first overcome defences erected by the host. The barriers to infection may be physical (eg. exoskeleton), physiological or biochemical (eg. host does not possess gut conditions compatible with parasite hatching and survival), and immunological (eg. the organism is phagocytosed by the immune cells).

This section will discuss the barriers presented by the host to infection and how the invading organisms might circumvent them.

PHYSICAL BARRIERS TO INFECTION

The arthropods present a hard chitinous exoskeleton, impenetrable to most organisms, except a few parasitic fungal species. Therefore, by far the most likely mode of infection for the arthropods is via the gut, especially true of animals which have adopted a scavenging lifestyle.

The digestive system of insects is divided into the chitinous and highly impenetrable foregut and hindgut, and the midgut which is lined with the peritrophic membrane and is more readily penetrated by a suitably armed parasite (see Chapman, 1985 for overview of insect gut structure). After ingestion, the parasites may be stored along with food items in the crop of the insect. Excessively long storage periods in the crop may cause parasite death through exhaustion of essential nutrients. Crop emptying rates are influenced by several factors including the osmotic pressure of the haemolymph, the amount of sugar present in the meal (Treherne, 1957) and the size of the meal (Englemann,1968). Gut transit times are variable between species (for

review see Dow, 1986) and this has important implications in assessing the suitability of the insect as a host.

In contrast with mammals, insects do not possess gut-associated lymphoid tissues yet colonisation of the gut with pathogenic organisms induces haemocytic immune responses, but no protective immunity (Glinski and Jarosz, 1986). This suggests first, that the insect immune system relies on a non-specific or innate immunity rather than an acquired immune response with a memory component, and second, that the immune system can be alerted by oral infection with potentially dangerous organisms. Since several helminths choose this route for infection, information on the immune response to stimulation via the gut is relevant to the natural situation. Indeed, recent reports suggest that immune stimulation occurs in cockroaches after feeding with the rat/beetle tapeworm, Hymenolepis diminuta (Holt, 1989a). This will be researched further in the present work.

IMMUNOLOGICAL BARRIERS TO INFECTION

After breaching the physical defences, the invader is confronted with the immune system of the invertebrate host. A well-adapted parasite will survive in the host until completion of the parasitic lifecycle, for example, until an opportunity arises to infect the final host. Precisely how these organisms live in contact with the immune system yet avoid attack is largely unknown. However, it is possible to predict that either recognition is avoided, by molecular mimicry for example, or the cellular activity is prevented, as in antigen shedding (see reviews by Bayne and Yoshino, 1989; Lackie, 1986b, Yoshino and Boswell, 1986).

The following sections will introduce the parasites used in this

thesis and their lifecycles and discuss how the immune system of the insect may respond after infection.

1.3. THE COCKROACH/PARASITE MODEL

Immunological analyses of host responses to parasites are best performed using well-defined experimental systems in which variables can be controlled. Although desirable, the practical difficulties of cellular immunology in insects as small as mosquitoes are simply enormous, although some authors have gallantly persevered (see Hall, 1983 for review of mosquito cell-mediated immunity). The cockroach, Periplaneta americana (Dictyoptera) is a useful model for studies on insect immunology because of its large haemolymph volume, the ease of laboratory maintenance and the wealth of information which exists on the biology of the animal. More specifically, the absence of allogeneic recognition and some combinations of xenogeneic recognition by the immune cells (Lackie, 1979; 1986a) provides the opportunity for experimental procedures not possible in higher animals.

The lifecycle of both Hymenolepis diminuta and Moniliformis moniliformis are readily maintained in the laboratory in their final host, the rat, and their intermediate insect hosts (see Figs. 1.1 and 1.2). Although both these parasites share the same final host and are, therefore, likely to occur concomitantly, Moniliformis naturally infects the cockroach whereas Hymenolepis does not. These systems provide useful models to study the discriminatory abilities and responsiveness of insect immunity.

By understanding the immune response which precedes elimination of Hymenolepis in the model system, a similar effector mechanism might be

enhanced and exploited in biological control programmes against parasites of medical importance. Also, understanding how Moniliformis avoids attack during development in the haemocoel may show the weaknesses in recognition or response in the insect immune system, of use in insect pest control using pathogens.

1.4. PARASITE SURVIVAL IN THE IMMUNE ENVIRONMENT

Parasitism is a balance between the benefit to the parasite in using host resources and the need for the host to avoid over-exploitation by the parasite. These factors work at the level of the individual as well as the population level. A well-adapted parasite is likely to cause the minimum of damage to the host while facilitating its own survival.

In the present study, host survival has been studied using the Moniliformis moniliformis/cockroach model.

The Lifecycle of Moniliformis moniliformis

Moniliformis moniliformis (previously known as Moniliformis dubius) is primarily a rodent-infecting acanthocephalan (Acanthocephala: Archiacanthocephala), although cases of human infection have been reported (Sahba et al., 1970). The cockroach, Periplaneta americana serves as an intermediate host, becoming infected under natural conditions by ingestion of the eggs (see Fig. 1.1 for lifecycle and Crompton and Nickol, 1985; Nicholas, 1967 for general biology). The dispersive stage or egg of Moniliformis was termed the shelled acanthor by Nicholas and Hynes (1963) because of the four surrounding protective envelopes. The egg is released by the adult female worm and is passed out in the faeces to be eaten by foraging cockroaches. Hatching of the acanthor is thought to be aided by a combination of larval enzymes,

rostellar hook activity (Edmonds,1966) and the ionic conditions of the host gut (Starling, 1985). After hatching, the larvae penetrate the cockroach gut, emerging in the haemocoel between 10 and 14 days later (Robinson and Strickland, 1969).

The parasite passes through several stages within the haemocoel of the cockroach, with completion of development occurring at around 7-8 weeks post infection depending on temperature (King and Robinson, 1967; Lackie, 1972a). Moore (1933) described the lifecycle of M. moniliformis. However, the nomenclature of King and Robinson (1967) will be used in this thesis.

Acanthor I - the hatched motile acanthor prior to penetration of the cockroach gut.

Acanthor II - the acanthor as it penetrates the midgut wall using the rostellar hooks and emerges in the haemocoel.

Acanthella I - the body spines are lost and the rostellar hooks are displaced from their anterior position.

Acanthella II - formation of the lemniscal nuclear ring.

Acanthella III - parasite increases in size. Larval body becomes Z-shaped within the envelope.

Acanthella IV - appearance of lemnisci.

Acanthella V - body becomes shortened, rounded and flattened.

Acanthella VI - invagination of the proboscis and neck.

Cystacanth - infective stage. Envelope enlarged. Body cap-shaped and shortened.

The Origin and Ultrastructure of the Envelope

There has been much controversy as to whether the cystacanth envelope is host or parasite derived. Crompton (1964), studying the development of the capsule around Polymorphus minutus in Gammarus pulex, suggested that the envelope arose as an eversion of the damaged gut serosa and

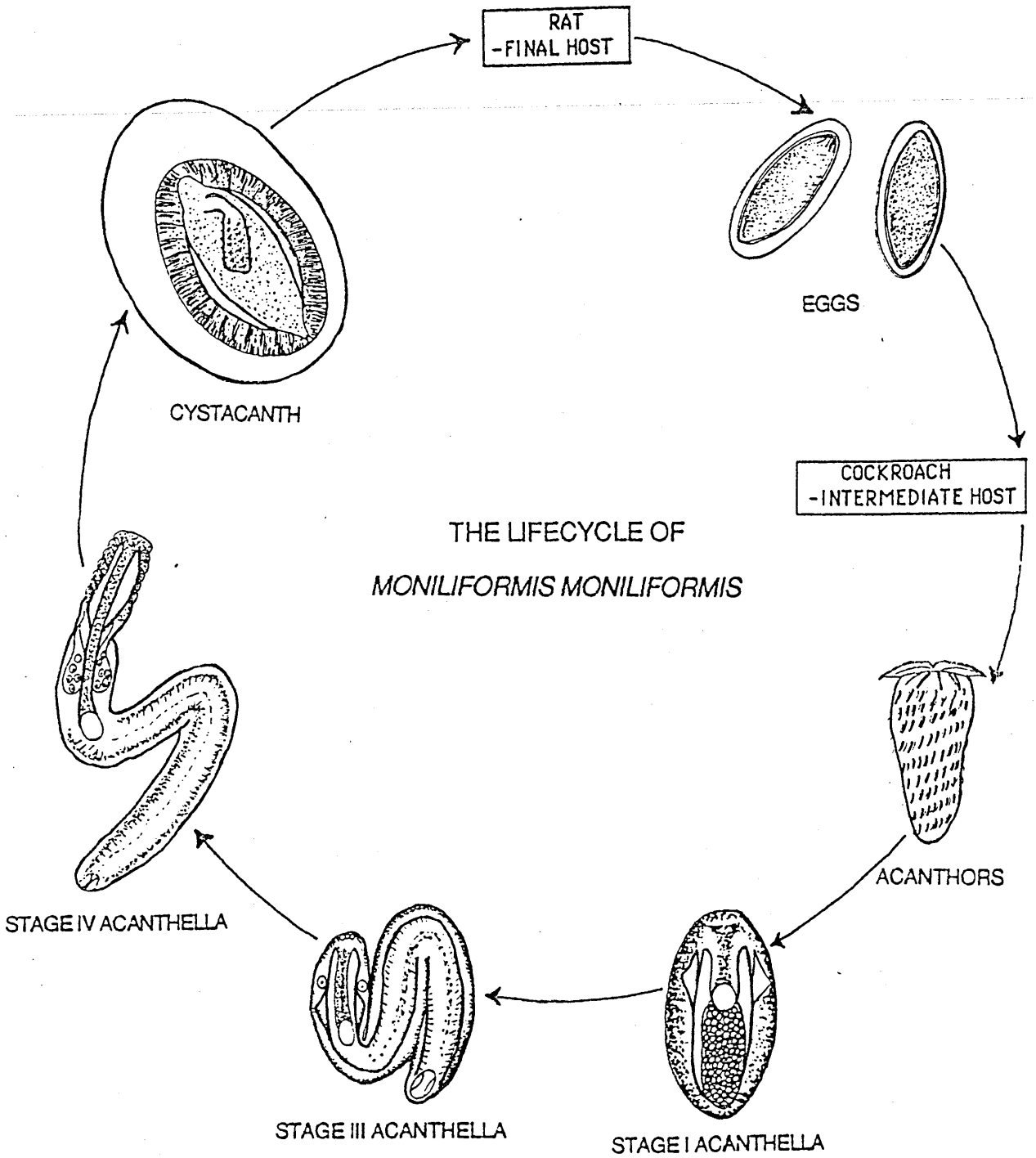


FIGURE 1.1 THE LIFECYCLE OF MONILIFORMIS MONILIFORMIS

The adult worms infect the rat gut and eggs are released along with the faeces. Cockroaches (Periplaneta americana) ingest the eggs which hatch in the midgut and the acanthors burrow across the gut wall. Development proceeds in the haemocoel. The cystacanth is infective to the rat final host (after King and Robinson, 1967).

that the wound-healing response of host haemocytes contributed to the structure of the parasite envelope. However, this seems unlikely since acanthors hatched in vitro developed normally when injected directly into the cockroach haemocoel, so by-passing gut penetration (Robinson and Strickland, 1969).

Examination of the ultrastructure of the developing envelope provided evidence that the envelope was of parasite origin (Wright and Lumsden, 1968) and arose from microvillar projections of the early acanthella tegument (Lackie and Rotheram, 1972: Rotheram and Crompton, 1972). The success of in vitro culture of hatched acanthors, in which the membraneous coat was formed, provided further evidence for the envelope being parasite-derived (Lackie, 1973). Finally, the absence of haemocytes on the surface of acanthellae envelopes implied that haemocytic material was not required for envelope development (Lackie and Rotheram, 1972). It is now widely accepted that the envelope is produced by the parasite.

Several authors have studied the ultrastructure of the larval envelope of M. moniliformis (Mercer and Nicholas, 1967; Lackie and Rotheram, 1972). The stage I acanthella is surrounded by a membranous and vesicular coat which may be formed from microvillate projections of the parasite tegument (Rotheram and Crompton, 1972). Haemocytes are found adhering to the surface of the membranous layer or embedded within it. By the cystacanth stage the envelope encloses the parasite very loosely, being separated from it by viscous material. The cystacanth envelope is formed of 3 layers, an inner loosely packed layer surrounded by a distinct amorphous layer and an outer layer containing loosely packed small vesicles.

The immune system of an animal may be suppressed either by reducing the availability of effectors, for example by lowering the cell count or by decreasing their effectiveness, for example by shedding molecules which block cellular binding sites so preventing cell adhesion to the invader.

By suppressing the host immune system, the parasite endangers its own survival since the host becomes more susceptible to opportunist infections. This is relevant for both the individual and the species since increased mortality of individual host animals due to parasitism may lead to a shortage of available hosts.

If the time for parasite development is short then immune suppression is a more viable proposition than for parasites with longer developmental periods. Moniliformis, which takes several weeks to develop in the insect haemocoel, has been suggested as suppressing the host cellular immune system (Lackie and Holt, 1988) even though to do so could be fatal for both organisms. Selective suppression of immune components could permit survival of one parasite yet still leave a relatively competent immune system. This hypothesis will be discussed later in this thesis.

Other examples of host immune suppression by parasites exist in the literature. Parasitic wasps, which lay their eggs in the larvae of other insects, have been reported to suppress the immune system of the host (Walker, 1959; Nappi and Streams, 1969), although the suppressive effect on the defences may be due to concurrent infection with viruses (Guzo and Stolz, 1987).

An alternative strategy to suppression is immune evasion, in which

the host does not recognise the invader as foreign. Either the parasite has evolved to look like "self" or it can adsorb host molecules as a disguise.

The larval envelope may have a protective role in defending the parasite against the insect immune system by mimicking cockroach tissues (Lackie, 1975). Robinson and Strickland (1969) found that de-enveloped larvae of M.moniliformis were encapsulated when injected into naive cockroaches. Further evidence for immune evasion was provided by the surprising discovery that H. diminuta larvae can survive within the cockroach host by entering the M. moniliformis envelope (Holt, 1989b). Lackie and Lackie (1979) tested the hypothesis that the envelope exhibited surface determinants which mimicked host tissues. Moniliformis larvae raised in locusts then transferred to cockroaches were not recognised as foreign. Since cockroaches can mount an immune response to haemolymph from locusts (Lackie, 1986a), it seemed unlikely that the parasite was disguised as the locust host. Rapid turnover of host-derived envelope molecules may replace the locust determinants before immunorecognition had occurred in the cockroach host.

Whatever the mechanism Moniliformis uses to survive long-term in the cockroach host, it seems not to be in operation immediately the acanthor enters the haemocoel. Newly emerged acanthors are encapsulated by blood cells and haemocytes aggregate around the sites of gut penetration (Robinson and Strickland, 1969).

Lackie (1986b) has reviewed immune evasion by helminthic larvae in some detail and the reader is referred to this work for more in-depth discussion. In the work reported here, the question of immune suppression or evasion by Moniliformis has been investigated using assays for immunocompetence.

1.5. PARASITE ELIMINATION FROM THE IMMUNE ENVIRONMENT

A parasite which is poorly adapted to the host may either inflict excess damage on the host or may fail to survive the onslaught of the immune defences. In the work presented here, Hymenolepis infections in the cockroach were used to model the defence response of an insect to a parasite which is susceptible to attack. By assaying immunocompetence in naive and Hymenolepis-infected animals an insight is gained into how the cellular immune defences respond to infection.

Hymenolepis diminuta

Hymenolepis diminuta (Platyhelminthes: Cestoda) has been found to hatch successfully within the cockroach gut but seems unable to penetrate the muscular midgut wall, becoming melanised and necrotic before complete penetration has occurred (R. Holt, unpublished observations). That the parasite is capable of hatching within the midgut suggests that the cockroach is physiologically an appropriate host and oncospheres hatched in vitro and injected directly into the haemocoel may develop within the cockroach, although the survival rate is low (Lackie, 1976). This implies that the gut is not the only barrier to Hymenolepis development within the cockroach.

Since H. diminuta is most often found melanised or encapsulated within the tissues or haemocoel of the cockroach, it is clear that the immune system is responsive to invasion by this parasite.

The Lifecycle of Hymenolepis diminuta

The cestode H. diminuta is parasitic in the rat gut. Infection occurs when the final host eats an infected intermediate host, commonly the

flour beetle, Tribolium confusum, and the worm achieves patency at approximately 17 days later. Infection of the intermediate host occurs after ingestion of gravid proglottids which detach from the posterior portion of the adult worm and pass along the rat intestine in the faeces (see Arai, 1980 for general biology of the tapeworm). Mechanical disruption of the outer shell of the egg by the insect mouthparts is required for hatching of the larva (Berntzen and Voge, 1965). After hatching, the oncosphere penetrates the anterior portion of the beetle midgut using the oncospheral hooks (Lethbridge, 1971) and an enzyme secreted from the penetration gland (Ubelaker, 1980).

Five stages have been recognised in the larval development of H. diminuta in the flour beetle T. confusum (Voge and Heyneman, 1957).

Stage 1 - 48hrs, the larva has a rounded appearance with the oncospheral hooks still visible.

Stage 2 - 48-72hr, a body cavity develops, oncospheral hooks move to posterior. Larva spheroidal.

Stage 3 - 72-120hr, larva begins to elongate and central cavity enlarges. Body divisions are distinguishable as tail, mid-body enclosing a large cavity, and the fore-body which will form the scolex.

Stage 4 - 120-140hr, the suckers become visible and the mid-body widens. The scolex is motile and withdrawn into the body cavity in the late stage 4.

Stage 5 - 144-192hr, the midbody and tail elongate. The scolex becomes more motile. At this stage the larva is infective to the primary host.

Host Response to Hymenolepis diminuta

Ubelaker, Cooper and Allison (1970) suggested a protective role against the host immune system for the outer layer of the H. diminuta cysticercoid, the microvillus layer. Vesicles formed from the microvillus layer were numerous in regions of the body wall in contact

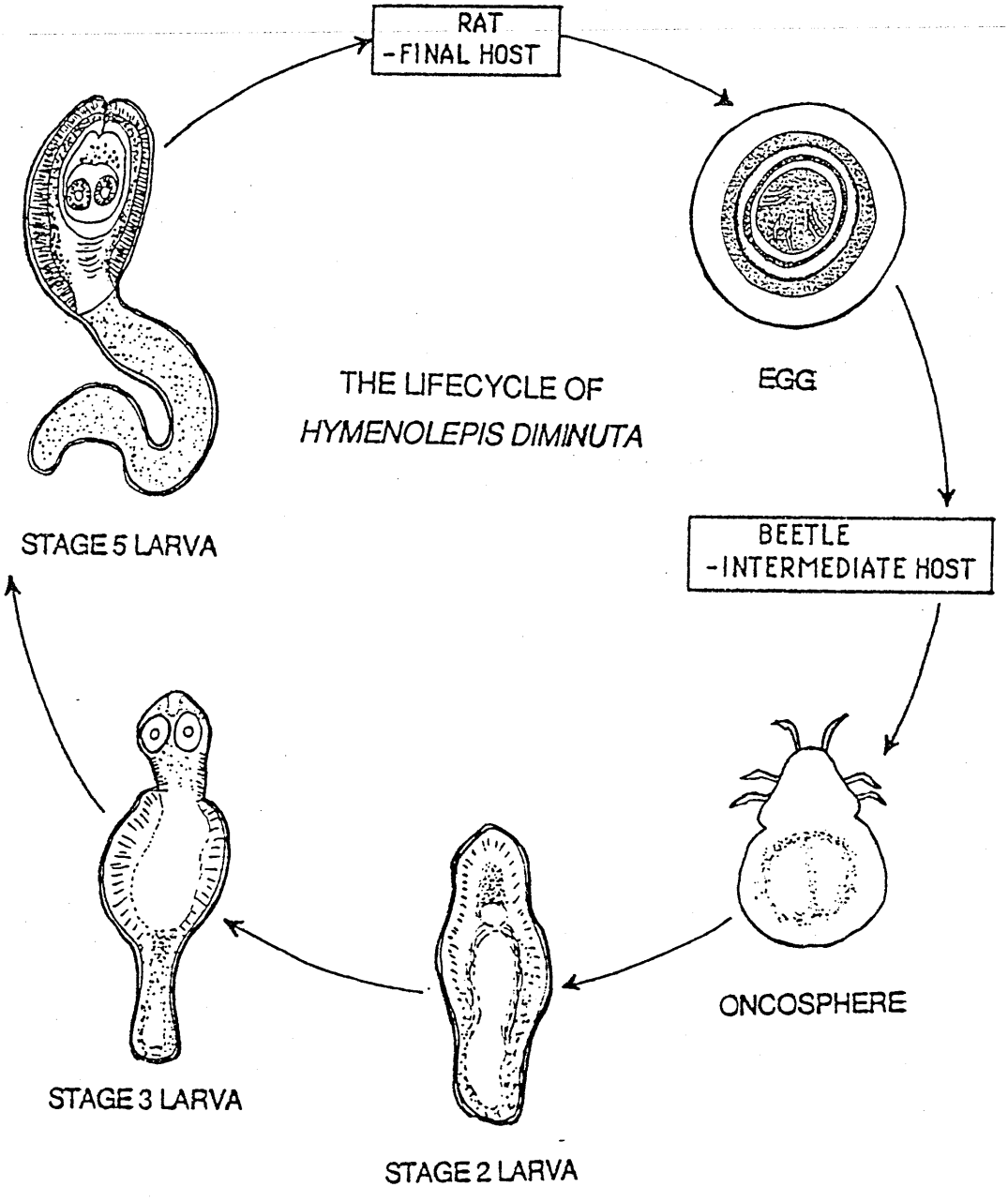


FIGURE 1.2 THE LIFECYCLE OF HYMENOLEPIS DIMINUTA

Adult worms infect the gut of the definitive host, the rat, and eggs pass out with the faeces. Flour beetles, Tribolium confusum, ingest the eggs which hatch and oncospheres penetrate the gut wall. Development proceeds in the haemocoel and the final stage, the cysticercoid, is infective to the rat host (after Voge and Heyneman, 1957).

with host haemocytes. The cells were often lysed and these researchers suggested that the vesicles contained material toxic to the cells.

In the flour beetle, the haemocytes appear capable of mounting an immune response to some hymenolepidid species. Heyneman and Voge (1971) compared the cellular response to H. diminuta, H. microstoma and H. citelli. Both H. diminuta and H.microstoma generally escaped encapsulation but H. citelli, which does not naturally infect flour beetles, became encapsulated.

Immunogenicity of hymenolepid cestodes in their hosts may be affected by changes in parasite antigenicity throughout development and differentiation (Ito et al., 1989). Although this may explain how the parasite escapes the host immune system, further work is needed to identify potential immunogens characteristic of the parasite stages.

1.6. THE IMMUNE RESPONSE

An efficient immune system can distinguish "self" from "non-self", remove potentially harmful organisms from circulation and destroy them.

The blood of higher vertebrates contains several readily distinguishable cell types eg. macrophages, lymphocytes which mediate specific and non-specific immunity. The plasma contains immunoglobulins, complement and other proteins involved in defence. The evolution of a complex immune system provides the higher animal with a flexible, specific response with a memory component, commonly known as adaptive or acquired immunity. In addition, vertebrates possess innate immunity, so-called because prior exposure to the agent does not heighten the response. Indeed, it is likely that acquired immunity evolved to enhance the effectiveness of the innate immunity. How

lower animals defend themselves from invasion has long been of minor importance to vertebrate immunologists and, compared with the vertebrates, the invertebrate defence system seems deceptively simple and primitive. Yet invertebrates provide an exciting and challenging opportunity to investigate an immune system which is devoid of classical humoral immunity ie. immunoglobulin and complement (Anderson, 1976) and most likely, memory. Although some authors (Karp and Rheins, 1980) have reported specific memory in invertebrate humoral defences, additional conclusive evidence is required. Thus, we have a perfect model in invertebrates to examine the functional importance of a cellular immune system which relies heavily on the components of innate immunity (see Ratcliffe et al., 1985; Lackie, 1986b; 1988a; 1988b for comprehensive reviews of invertebrate immunology).

1.6.1. HUMORAL IMMUNITY

This topic has been reviewed by Boman and Hultmark (1981: 1987) and the reader is referred to this work for detailed discussion.

The Anti-bacterial Proteins

Humoral antibacterial activity can be induced in some insect species in response to infection and can be attributed to humoral proteins. Most notable and best researched are the inducible anti-bacterial proteins of the holometabolic Lepidoptera. Larvae of Hyalophora cecropia provide a useful model because of their ample blood volume and their ability to combat infection while in diapause. After wounding (Postlethwait et al., 1988) or infection with live non-pathogenic bacteria or killed pathogens, the protein composition of the haemolymph is modified by the appearance of proteins which have been newly synthesised in the fat

body (Trenczek, 1986) and this is associated with a non-specific protection against challenge. Around 15-20 inducible proteins have been found in the haemolymph of H. cecropia. Several of these molecules have now been purified and their molecular structure determined (for reviews see Hultmark et al., 1983; Boman, 1986). Three groups of antibacterial proteins are recognised on the basis of their structures and activities; the cecropins, the attacins and lysozyme.

Most of the antibacterial activity of the inducible proteins can be attributed to small (4Kd) basic proteins, the cecropins, previously named P9 (see Boman, 1986 for review). These proteins are effective against both Gram positive and Gram negative bacteria (Hoffman et al., 1981) and a detergent-like activity against the membrane has been proposed because of their structure (Boman, 1986).

Attacins, previously known as P5, have been found in the haemolymph of cecropia (Hultmark et al., 1983), Glossina morsitans (Kaaya et al., 1987) and Drosophila (Flyg et al., 1987). The attacins of H. cecropia are 6 closely related 20-23Kd proteins which may be sub-divided into 2 types according to their molecular structure. Attacins A-D are basic whereas attacins E and F have acidic properties. The attacins appear to affect cell division and are effective against a limited number of Gram negative bacteria in a dose-dependent manner (Hultmark et al., 1983; Kaaya et al., 1987).

Originally cecropins and attacins were thought to occur exclusively in the Holometabola eg. Lepidoptera (Hultmark et al., 1983; Hoffman et al., 1981) and Diptera (Kaaya et al., 1987; Flyg et al., 1987). However, cecropin-like molecules with bactericidal properties have recently been found in the bug, Rhodnius prolixus (De Azambuja et al.,

1986). Although inducible proteins are detectable in the Orthoptera, their antibacterial activity can be assigned to lysozyme (Schneider, 1985).

Lysozyme is one of the antibacterial proteins found in immune haemolymph (Mohrig and Messner, 1968) which has since been purified and characterised (Powning and Davidson, 1973; Zachary and Hoffman, 1984; Schneider, 1985). This lytic enzyme acts specifically on the β1,4-linkage between N-acetylglucosamine and N-acetylmuramic acid of peptidoglycan in the cell walls of Gram positive bacteria. Lysozyme is induced by wounding, saline injection, whole heat-killed bacteria (Jarosz and spiewak, 1979), bacterial lipopolysaccharide (LPS), abiotic particles (Anderson and Cook, 1979; Mohrig and Messner, 1968) and bacterial cell walls (Powning and Davidson, 1973). The flagellates, Trypanosoma brucei and Leishmania hertigi induces lysozyme production in the haemolymph of the cockroach, P. americana (Ingram et al., 1984) and lysozyme has been found to be effective in killing many intracellular organisms.

Lysozyme was originally assigned a central role in humoral immunity by some authors (Mohrig and Messner, 1968), while others (Chadwick, 1970; Tripp, 1975) were more reserved about the biological function of this enzyme in invertebrate immunity. Multiple lysozymes were found in the cricket, Gryllus bimaculatus, a hemimetabolic insect which lacks cecropin-like activity (Schneider, 1985). However, insects can eliminate bacteria resistant to lysozyme activity (Boman, 1982) so lysozyme must work in concert with the other bacterial proteins. Although lysozyme is induced by colonisation of Galleria mellonella gut with pathogenic bacteria, it does not confer protective immunity to challenged animals (Glinski and Jarosz, 1986). The primary function of

haemolymph lysozyme may not be protective, therefore, but rather degradative, acting on the cell wall of organisms killed by the other antibacterial proteins.

P4 is the major induced protein of Hyalophora cecropia, yet its function in the immune response remains to be determined. Two forms of P4 exist, both with a molecular weight of 48Kd, and these have been isolated and characterised (Andersson and Steiner, 1987). This protein does not exhibit antibacterial properties, yet the haemolymph P4 titre increases with bacterial infection.

Other antibacterial proteins have been isolated from dipteran flies, including Sarcophaga peregrina (Okado and Natori, 1985) and Drosophila melanogaster (Robertson and Postlethwait, 1986). More recently, Lambert et al. (1989) have isolated and characterised two 4Kd peptides, which they term "insect defensins", from the dipteran, Phormia terranovae. This insect lacks haemolymph lysozyme yet exhibits potent anti-Gram positive activity, now attributed to the defensins. These molecules are not analagous to any other known antibacterial protein of insects, yet they have many structural similarities to cationic peptides of vertebrate macrophages and neutrophils.

Humoral Encapsulation

Acellular, or humoral capsules are usually found in insects in which cellular immunity is limited because of a low haemogram and occur in dipteran flies in response to nematodes, fungi and bacteria (see Gotz, 1986a, for review) and non-living material (Gotz, 1986b). Humoral encapsulation of bacteria has been observed in vitro and the efficiency and speed of encapsulation is independent of the bacterial type (Gotz

et al., 1987).

Melanin seems to be a constituent of humoral capsules since phenoloxidase inhibitors prevent hardening of the capsule (Vey and Gotz, 1975). Interestingly, Chen and Laurence (1985) have reported that the pigmented layer, the humoral capsule, is deposited around microfilariae of Brugia pahangi before the onset of cellular encapsulation in the anopheline mosquito host. These authors suggest that phenolic compounds produced during the melanotic capsule formation are toxic and that the subsequent cellular capsule might serve to isolate these destructive molecules and protect the host tissues.

MELANISATION AND PROPHENOLOXIDASE

Melanin is often associated with the cellular and humoral immunity of invertebrates since it is found at the centre of nodules and is a component of humoral (Gotz, 1973: 1986b) and cellular capsules (Nappi, 1973). Consequently, this substance and the precursor enzymes required for its production, such as prophenoloxidase, have been thoroughly investigated because of their importance in the immune system.

Phenoloxidase is the end-product of the activation of prophenoloxidase (Soderhall, 1982), via a complex biological cascade pathway (Ashida and Soderhall, 1984; Soderhall and Smith, 1986). Prophenoloxidase is an intracellular, inactive pro-enzyme and several serine proteases and calcium ions are involved in the activation process. A number of non-self molecules, such as bacterial lipopolysaccharides and β1,3-glucans (Soderhall and Hall, 1984; for reviews see Soderhall, 1982; Soderhall and Smith, 1986; Brehelin et al, 1989) can trigger prophenoloxidase activation. Huxham and Lackie (1986) have described a simple in vitro method for testing the stimulatory or

inhibitory effects of various molecules on the prophenoloxidase pathway. Some similarities between the prophenoloxidase system and mammalian complement do exist: both can be activated by zymosan and bacterial lipopolysaccharide (Czop and Austen, 1985) and both are activated by a cascade reaction but whether the two are functionally or phylogenetically analagous is debatable.

That phenoloxidase is involved in cellular immunity has long been suspected but the exact role played by the enzyme has proved elusive (for review see Soderhall and Smith, 1986). However, there is an increasing body of evidence which suggests that phenoloxidase performs other functions in the immune response apart from melanisation. Soderhall (1982) has suggested that the phenoloxidase pathway may act as a general recognition mechanism in invertebrate immunity and could initiate and co-ordinate cellular events.

More detailed investigations using *in vitro* techniques and separated cell populations (Soderhall *et al.*, 1986) has provided much information on the significance of prophenoloxidase in cell behaviour. Smith and Soderhall (1983a; 1984) found that the phagocytic capacity of crab and crayfish haemocytes *in vitro* could be enhanced by the inclusion of prophenoloxidase activators (laminarin and zymosan) in the cell culture medium. An opsonic role for the proteins derived from the prophenoloxidase cascade was suggested by these authors. In a similar series of experiments, phagocytosis of bacteria by insect haemocytes was greater after incubation with laminarin (Leonard *et al.*, 1985) and bacterial endotoxin, both of which activate prophenoloxidase (Ratcliffe *et al.*, 1984).

However, opsonisation of bacteria is not the sole explanation for

improved phagocytic clearance in this situation. Activated prophenoloxidase stimulates chemokinetic locomotion in cockroach haemocytes *in vitro* (Takle and Lackie, 1986) and with enhanced locomotion, immune surveillance may also improve. Also, Dularay and Lackie (1985) found that activated components of the prophenoloxidase system when bound to negatively charged ion-exchange beads did not alter the ability of locust cells to encapsulate these beads suggesting that the prophenoloxidase pathway does not have an opsonic function.

However, prophenoloxidase may initiate and co-ordinate cellular events in the immune system. A peptide which mediates cell adhesion has recently been purified and characterised (Johansson and Soderhall, 1988) and its activity is concurrent with prophenoloxidase activation. Johansson and Soderhall (1989) have found that this peptide also causes degranulation of crayfish granular cells. These authors propose a model for encapsulation in which the peptide mediates degranulation of granular cells and attachment of cells to the foreign material. In support of this model, Persson *et al.* (1987) found that encapsulation is prevented if haemocyte degranulation is inhibited.

It is clear, therefore, that the prophenoloxidase pathway mediates many aspects of cell behaviour during the immune response. However, we should be careful about over-emphasising prophenoloxidase activation to the detriment of other aspects of the immune machinery.

1.6.2. CELLULAR IMMUNITY

The blood cells, or haemocytes, circulate freely in the blood space of the insect and are the effectors of the defence reaction.

CLASSIFICATION OF HAEMOCYTES

Identification and classification of blood cells in the insect is difficult because of inconsistencies in the methods used for collection and preparation and the morphological changes which haemocytes undergo on storage and culture.

Brehelin and Zachary (1986) examined the haemocyte terminology and classified nine types of insect haemocytes, emphasising the importance of cell granules for identification. The plasmatocytes, for instance, were termed agranular cells although other authors (Lackie, Takle and Tetley, 1985; Huxham and Lackie, 1988) consider that plasmatocytes may contain granules, at least in some insects. Price and Ratcliffe (1974) used amoeboid movement as an indicator for the plasmatocyte cell type. The reader is referred to a recent review by Lackie (1988) for further discussion of insect haemocyte types. The terminology used in the present study for P. americana haemocytes is that of Lackie et al. (1985).

Prohaemocytes - These cells are present in low numbers in almost all insects. The prohaemocytes are small rounded or oval cells (6-13μm diameter) with granular cytoplasm and often indistinguishable from the plasmatocytes. They exhibit a high mitotic index, unusual in haemocytes, and are considered to be the stem cells of circulating haemocytes.

Plasmatocytes - Granular inclusions (0.5 - 1.5μm diameter) are present in the cytoplasm of these cells. These large polymorphic cells are thought to be present in all insects. Plasmatocytes have a high phagocytic capacity and marked ability to spread, although they usually appear spindle-shaped *in vivo*. These cells perform a function in nodule and capsule formation.

Granulocytes - Unlike the plasmatocytes, these cells appear to be incapable of extensive spreading, although cytoplasmic extensions anchor the cell to the substratum. The cytoplasm contains several types of granules, both structured and unstructured (Goffinet and Gregoire, 1975). Due to the morphological similarity of these cells to the plasmatocytes, some authors consider them to be the same cell type (Ravindranath, 1978, Lackie *et al.*, 1985). In this study, granulocytes are considered to be a behavioural, and possibly functional, subclass of plasmatocytes.

Coagulocytes - These are rounded cells of 9 -14μm diameter which often lyse *in vitro* (Lackie *et al.*, 1985).

Also present but rarely seen in the circulating haemogram are the oenocytoids, spherulocytes and adipohaemocytes. The oenocytoids contain homogeneous cytoplasm whereas the cytoplasm of the spherulocytes has numerous granules. The adipohaemocytes are distinguishable by the fat droplets in the cytoplasm and similarities between these cells and fat body cells have been noted (Price and Ratcliffe, 1974).

CELLULAR DEFENCE REACTIONS

The blood cells deal with invasion in three ways depending on the nature of the foreign material. Small particles are engulfed by

phagocytes or immobilised in nodules if the infection exceeds the phagocytic capabilities of the blood cells. Larger objects are trapped in cellular capsules.

PHAGOCYTOSIS

Phagocytosis is the ingestion of particles by single cells and is probably, phylogenetically, the oldest and most fundamental defence mechanism of the host against invaders. Phagocytosis requires recognition of and contact with the foreign material before engulfment and killing can occur.

In the insects, the plasmatocytes are the main phagocytic cells (Brehelin and Hoffman, 1980; Guzo and Stoltz, 1987). Mammalian phagocytes have a variety of specialised membrane receptors to enhance phagocytic uptake, the most important of which recognise and bind antibody molecules coating the invading organism. Since invertebrates are lacking in immunoglobulins, lectins have been proposed as opsonins (see below for discussion).

Although phagocytosis is readily stimulated by particulate material, non-specific stimuli such as saline injection (Gunnarsson, 1988a) probably enhance phagocytic uptake as a result of a wound response. Likewise, the β1,3-glucans from yeast cell walls (Bacon *et al.*, 1969) vastly increase phagocytic clearance of target particles *in vivo* (Gunnarsson, 1988a) and *in vitro* (Ratcliffe *et al.*, 1984; Huxham and Lackie, 1988). Evidence presented in this study (Chapter 3) shows that oral infection with a helminthic parasite also increases phagocytosis and this could reflect improved surveillance and responsiveness by the defence system after infection.

NODULE FORMATION

Nodule formation is the term used by invertebrate haematologists to describe the process of cell contact and adhesion resulting in cellular aggregates. Numerous authors (Salt,1970; Ratcliffe and Gagen, 1976; Ratcliffe and Walters, 1983; Rahmet-Alla and Rowley, 1989) have reported the formation of nodules as a response to injected micro-organisms. However, soluble foreign molecules injected into the haemocoel also induce nodule formation (Smith, Soderhall and Hamilton, 1984: Gunnarsson and Lackie, 1985) suggesting that cell activation can occur by binding of soluble molecules to cell surface receptors. Ratcliffe and Walters (1983) found that nodule formation was particularly effective in removing large numbers ($>10^3$) of pathogenic and non-pathogenic bacteria from circulation. Nodules are formed in Galleria mellonella and Pieris brassicae by injection of live and killed Bacillus cereus (Ratcliffe and Gagen, 1976). Nodules are formed very rapidly in response to infection and both plasmatocytes and granulocytes participate, the centrally located granular cells quickly becoming necrotic and melanised while the plasmatocytes flatten forming a sheath around the melanised core. Melanised nodules are often found stuck to the internal organs and fat body of insects injected with bacteria (Ratcliffe and Gagen, 1976).

Gunnarsson and Lackie (1985) tested the efficacy of several microbial substances at inducing nodule formation in Schistocerca gregaria and found that fungal spores, bacterial lipopolysaccaride (LPS), β1,3-glucans (zymosan and laminarin) stimulated cell aggregation whereas the β1,6-glucan, dextran, did not. Peptidoglycan is a major component of the cell walls of Gram-positive bacteria and when the soluble

peptidoglycan layer is removed by lysozyme pretreatment the stimulatory effect on nodule formation is reduced (Brookman et al., 1989). These authors also showed that capsular polysaccharide would elicit nodule formation. The responsiveness of the immune cells to several bacterial components allows the animals to mount an immune response to the majority of the bacteria found in the natural environment.

The ability of molecules to stimulate nodule formation has been suggested as correlating with the ability to activate the prophenoloxidase pathway (Brookman et al., 1989). However, Dularay and Lackie (1985) found no dependence on prophenoloxidase activation for encapsulation in S. gregaria, since normally non-encapsulated beads did not become encapsulated even after incubation with haemocyte lysate containing preactivated phenoloxidase.

CELLULAR ENCAPSULATION

Encapsulation is the "walling-off" of substances or particles too large to be dealt with by phagocytosis and nodule formation. Helminthic parasites and fungi are commonly dealt with by encapsulation in invertebrates, which may be either acellular or cellular. Chapter 4 investigates cellular capsule formation in vivo and in vitro by haemocytes of parasitised cockroaches.

Encapsulation is perhaps one of the most fascinating defence phenomena to occur in invertebrates since it encompasses all aspects of cell behaviour (see Gotz, 1986b; Lackie, 1988a for reviews). To encapsulate, the cells of the immune system must recognise the invader, adhere and spread over the surface and interact with other cells to regulate the event so as to maintain a relatively local response. An

interesting phenomenon of capsule formation is the continued adhesion of cells to the foreign object even after the surface is covered. Those cells which adhere later are not exposed to the original surface and, therefore, must receive the stimulus to adhere indirectly via the encapsulating cells. Salt (1970) rejected the likelihood that factors diffused across the capsule since inert abiotic implants also stimulated thick capsules. Cessation of capsule formation is, therefore, likely to occur when the capsular cells are no longer "sticky" to the circulating cells (see Ratner and Vinson, 1983a for discussion of factors controlling encapsulation reactions).

Not all foreign material introduced into the haemocoel is encapsulated. Some parasites can avoid haemocytic encapsulation eg. Moniliformis. Plasmatocytes and/or granulocytes are involved in capsule formation (Ratcliffe and Rowley, 1979). However, other cell types eg. coagulocytes may play a part in initiation or cessation of cellular encapsulation. Persson et al. (1987) found that isolated semi-granular cells of the crayfish, Astacus leptodactylus formed apparently normal capsules whereas the granular cells, in contrast, were unable to form the layers of flattened cells characteristic of in vivo encapsulation indicating that encapsulation requires cell co-operation.

Capsule Ultrastructure

Details of capsule structure and formation in insects have been elucidated by scanning (Lackie et al., 1985) and transmission electron microscope studies (Grimstone et al., 1967; Gupta and Han, 1988). The formation of capsules can be envisaged as a multi-stage sequence. In the early stages, haemocytes adhere loosely to form a capsule with a irregular, narrow outline. Later, the capsule becomes thicker and more

compact (Salt, 1970) and plasmatocytes adhere and flatten forming interdigitating layers exhibiting cell junctions (Baerwald, 1979; Gupta and Han, 1988). By around 18-24hr after implantation, the capsule is thick, with a regular outline and recruitment of haemocytes has ceased by between 18-24hr and the central core is often melanised (Takle, 1986). Finally, capsules are covered with an extracellular material which contains glycosaminoglycans (acid mucopolysaccharides) (Lackie et al., 1985). A similar material is deposited between the flattened plasmatocytes (Grimstone et al., 1967).

Cell Spreading and Encapsulation

The relevance of microtubule and microfilament assembly in haemocytes involved in capsule formation was studied by Davies and Preston (1987). These authors suggested that the initial adhesion is independent of normal actin microfilament function although the second phase of cell spreading and compaction of the capsule depends on actin polymerisation and is energy requiring (Ratner and Vinson, 1983a). Johansson and Soderhall (1988) found that cell spreading on a 2-D substratum was inhibited by metabolic poisons, although initial cell attachment was unaffected. To survive in the haemocoel, well adapted parasites might affect the ability of plasmatocytes to spread and encapsulate in order to debilitate the immune system.

The thickness of the capsule depends upon the number of available blood cells (Lackie et al., 1985) and the physical (Lackie, 1983; Takle, 1986) and molecular (Lackie and Vasta, 1988) nature of the foreign surface. The following section will deal with cell adhesion and the factors which influence the adhesiveness of a substratum.

ADHESION

Cell adhesion is essential for many biological processes eg. cell locomotion and migration, blood cell margination and cancer metastasis, regeneration and embryogenesis. Similarly, mutual adhesiveness and adhesion to substrata is central to defence reactions by invertebrate blood cells.

Mechanisms of Cell Adhesion

When considering adhesion to a surface, there are many aspects to be considered including the potential of the substratum and its physical topography, the protein and ion composition of the medium and the presence of specific adhesion molecules eg. lectins, on the surface.

Several hypotheses have been advanced to explain cell adhesiveness and these are usually divided into two major categories. The first is possibly responsible for generalised adhesiveness and involves the surface properties, or physico-chemical properties, of the substratum. The second is more specific and involves receptor-ligand complexes.

1. Physico-chemical properties

The DLVO theory was proposed by Derjaguin and Landau (1941) and Verwey and Overbeek (1948). This originated from theoretical work performed on colloidal science and was applied to cells because of the physical similarity between cells and spherical, negatively charged colloid particles. The DLVO theory considers two types of forces; the electrostatic charge-repulsion force and the attractive van der Waals-type force, the London dispersion force (see Napper, 1967 for review).

If the two surfaces carry an opposite charge then attraction will

occur. The London dispersion force is a force of attraction which arises from the electron fluctuations of similar molecules within the two approaching surfaces. This attraction increases as the surfaces become closer.

Almost all cells possess a net negative charge so that a cell approaching a surface with the same charge will be repulsed by electrostatic forces (Overbeek, 1952). The charge on the cell surface is associated with the dissociation of surface molecules, normally the terminal carboxyl groups of sugars. The magnitude of the repulsive force is dependent upon the surface potentials, the ionic strength of the medium and the distance between the two approaching surfaces.

Using the DLVO theory, two separation distances can be derived, the primary minimum and the secondary minimum, at which net attraction occurs. The primary minimum occurs when the separation distance between surfaces is very small whereas the secondary minimum occurs when the particles are separated by 5-10nm. These are separated by a repulsive maximum. Adhesion of particles in the primary minimum is very stable whereas particles attracted via the weaker secondary minimum remain in equilibrium with the surrounding bulk phase.

In summary, the DLVO theory states that the total interaction energy of two smooth particles at close separations is determined by the sum of the van der Waals attractive energy (London Dispersion Forces) and the electrostatic energy, which is usually repulsive.

Surface Charge and Haemocyte Adhesion

Attempts to clarify the process of encapsulation by defining the surface properties of the target has led authors to investigate the adhesion to ion-exchange beads and polystyrene surfaces of different

charge. The effect of surface charge on haemocyte behaviour was discovered by chance during an investigation into aggregation of fat body cells (Walters and Williams, 1966). As an *in vitro* model, the fat body cells were replaced with Sephadex beads. The plasmatocytes adhered to and induced clumping of the positively charged beads whereas the neutral and negatively charged beads remained cell-free. This report of a differential effect of substratum charge on haemocyte adhesion initiated further research. The *in vitro* model used by these researchers has been much modified and elaborated and now forms the basis for elegant studies on *in vitro* encapsulation (Davies *et al.*, 1988; Davies and Preston, 1987; Ratner and Vinson, 1983a). In a similar but later study, Dunphy and Nolan (1980b) discovered that haemocytes of the Eastern hemlock looper, *Lambdina fiscellaria fiscellaria*, adhered exclusively to the positively charged DEAE-Sephadex, the negatively charged CM-Sephadex remaining devoid of cells.

Lackie (1983) looked at the effects of surface charge and wettability on adhesion and encapsulation by haemocytes of the cockroach, *P. americana* and the locust, *S. gregaria*. Regardless of the bead charge, the beads recovered from *S. gregaria* were less well encapsulated than those from *P. americana*. However, this difference was most marked for the negatively charged (amino groups) CM-Sepharose which, surprisingly, remained totally unencapsulated by the locust haemocytes.

Acid pretreatment of polystyrene, to increase the negative surface charge and wettability, induced an increase in the ability of the cockroach haemocytes to encapsulate beads *in vivo* and adhere *in vitro*. Locust haemocytes did not encapsulate either surface well. This suggested that the cellular immune system of *Schistocerca* may be poorer

at discriminating " self" from "non-self", as evidenced by their inability to respond to intrahaemocoelic parasites (Lackie, 1981a).

Using cell electrophoresis and the electron microscope visualisation of cationised ferritin binding, Takle and Lackie (1985) found that locust cells carry a larger net negative charge than cockroach cells, although the cell populations were heterogeneous with respect to the surface charge. Since locust cells carry a high negative charge the repulsive force exerted on contact with a similarly charged substratum may explain their inability to encapsulate negatively charged abiotic particles and respond adequately to invading micro-organisms. However, the surface charge interactions between parasites and haemocytes cannot wholly explain host susceptibility. These authors studied the infectivity of Trypanosoma rangeli for 4 insect species. Rhodnius prolixus, the usual intermediate host, is susceptible to T. rangeli infection yet haemocytes from Rhodnius carry the same net negative charge as the non-susceptible S. gregaria (Takle and Lackie, 1987).

2. Receptor - Ligand Binding (Lectins)

Proteins which have the ability to agglutinate cells by binding specifically to the cell surface saccharide molecules are found in both plants and animals and are referred to as lectins (see Renwrantz, 1986 for review of lectins in molluscs and arthropods). Lectins are found in all invertebrate phyla investigated (Yeaton, 1981; Vasta and Marchalonis, 1985), and although detailed information of their structure and carbohydrate-binding specificities exist, their function in the immune response is still poorly understood. The interaction between lectins and cell surface glycoproteins and polysaccharides in invertebrates may be analagous to the antigen-antibody recognition and

binding of vertebrates. In invertebrates, which lack immunoglobulin, humoral lectins may perform certain immunological tasks such as mediating non-self recognition and acting as opsonins to facilitate phagocytosis (Renwrantz and Stahmer, 1983). Because they bind to carbohydrate moieties, humoral lectins can agglutinate and immobilise invading micro-organisms, so aiding the subsequent cellular attack. Komano and Natori (1985) have reported that the agglutinin found in larvae of Sarcophaga peregrina is involved in clearing the haemolymph of sheep red blood cells.

Haemocytes express membrane bound lectins (Lackie and Vasta, 1986) and these molecules may be responsible for cell co-operation and identification of foreign or damaged material. Furthermore, since haemocytes are heterogeneous with respect to membrane lectin (Lackie and Vasta, 1988; Parrinello and Arizza, 1988) this may reflect functional differences between cell subpopulations in the immune response.

Chapter 5 investigates the role of cockroach haemocyte surface lectin in cell adhesion and non-self recognition in vitro by blood cells of the American cockroach, P. americana.

Prophenoloxidase and Adhesion

Recent research on encapsulation is more biochemically rather than ultrastructurally biased, with the isolation and characterisation of adhesion promoting factors in crustaceans (Johansson and Soderhall, 1988) and insects (Davies et al., 1988). A stable 76 kD protein has been isolated from the haemocyte lysate of granular cells which contained activated prophenoloxidase (Johansson and Soderhall, 1988).

No cell adhesive activity was found in non-activated lysates and secreted material from the granular cells, when preactivated with laminarin, induced adherence indicating that the protein occurs on activation of prophenoloxidase.

KILLING AND DIGESTION

Despite many years of research into invertebrate immune systems, we remain remarkably ignorant of how these animals kill invading organisms. Not surprisingly, the deposition of melanin at the centre of nodules and capsules has led some authors to ascribe a role for prophenoloxidase in killing (Soderhall and Ajaxon, 1982; Ratcliffe, 1986). Most often associated with this has been the production of toxic quinones during extracellular melanin formation (Chen and Laurence, 1985). However, whether melanisation alone is responsible for killing is doubtful since organisms can survive and develop in melanised humoral capsules (Gotz, 1986b). Therefore, the production of a melanised capsule may simply be a blockade strategy. Consequently, other candidates for killing mechanisms have been researched including lysozyme and the inducible antibacterial proteins which are active against Gram-positive and Gram-negative bacteria (see section 1.6.1). Relatively little attention has been paid to the killing and digestion of helminthic parasites in arthropods. In chapter 6, the intrahaemocytic enzymic changes (acid phosphatase and peroxidase) associated with infection with H. diminuta and M. moniliformis are reported.

Acid phosphatase has been reported in the haemocytes of molluscs (Yoshino and Cheng, 1976; Moore and Eble, 1977; Cooper-Willis, 1979; Sminia and Barendsen, 1980; Dikkeboom et al., 1984; Moore and Gelder,

1985), crustaceans (Hose et al., 1987) and insects (Chain and Anderson, 1983b; Walters and Ratcliffe, 1981). This is an acidic lysosomal enzyme which acts on phosphate bonds to degrade material in the secondary lysosome. Under neutral and alkaline conditions, this enzyme is inactivated to avoid degradation of the cytosol. However, acid phosphatase has been reported to be secreted under certain conditions (Cheng and Mohandas, 1985) although the function of extracellularly secreted acid phosphatase remains unclear since the haemolymph pH falls around neutral.

Levels of lysosomal enzymes, specifically acid phosphatase, in the haemocytes and haemolymph increase with bacterial challenge (Cooper-Willis, 1979; Cheng and Mohandas, 1985) and some authors consider that host resistance to infection may be attributed to high levels of lysosomal enzymes (Dikkeboom et al., 1984; Granath and Yoshino, 1983). However, peroxidase may be more important than acid phosphatase with respect to host immunity since this enzyme is central to killing by vertebrate phagocytes.

Mammalian leucocytes, namely macrophages and neutrophils, undergo increased oxygen consumption, known as the respiratory burst, during phagocytic uptake and in response to particulate and soluble stimulatory molecules eg. zymosan, phorbol myristate acetate (PMA) and formyl peptides (fMLP). This is associated with the production of toxic oxygen metabolites eg. superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH.) and singlet oxygen ($^1O_2$). These oxygen species are active against bacteria, yeasts and viruses. Several techniques are available for the study of the respiratory burst, including chemiluminescence, the recording of oxygen consumption, and

cytochemical detection of oxygen species eg. nitroblue tetrazolium (NBT) and diaminobenzidine (DAB). The respiratory burst and the presence of peroxidase has been demonstrated in the haemocytes of the molluscs Biomphalaria glabrata (Granath and Yoshino, 1983; Sminia and Barendsen, 1980), Mercenaria mercenaria (Gelder and Moore, 1986) and Lymnaea stagnalis (Dikkeboom et al., 1984, 1987). Hydrogen peroxide evolution has been detected in vitro by haemocytes of the scallop, Patinopectin yessoensis (Nakamura, 1985). However, this antimicrobial system has not been demonstrated conclusively in insect haemocytes (Anderson et al., 1973; 1976), although Misko (1972) has visualised peroxidase in cockroach blood cells.

Immune killing by insects might then be performed by a multiplicity of factors, including the prophenoloxidase pathway, the lysosomal enzymes and the inducible anti-bacterial proteins. Whether these factors might be used together in one offensive or used selectively, depending on the nature and intensity of the infection, is an interesting question which deserves more attention.

In the present study (Chapter 6), the cytochemistry (acid phosphatase, peroxidase and lysozyme) of haemocytes from P. americana has been studied after immune stimulation with Zymosan A supernatant and after infection with Hymenolepis and Moniliformis. These studies may provide an insight into the general functioning of the invertebrate immune system.

1.7. OUTLINE OF THE AIMS OF THIS THESIS

Given that Moniliformis larvae survive in the cockroach host whereas larvae of Hymenolepis are destroyed, the overall aim of the project was to identify changes in the host immune system during infection and to

relate these to parasite survival.

Several assays for cell behaviour and biochemistry have been used in this work to compare immune reactivity during infection with the two parasites and soluble stimulatory molecules (β1,3-glucans).

The aims of the project can be presented, thus;

1. To determine the changes in the haemogram after infection with the parasites and stimulation with β1,3-glucans.

2. To find how the cockroach immune system responds to infection by assessing the responsiveness of the blood cells using cell behavioural assays.

3. To investigate the basis of recognition of foreignness by the haemocytes, with emphasis on receptor-ligand binding.

4. To elucidate the mechanisms of immune killing by the haemocytes.

5. To investigate the cytochemistry of the haemocytes, with a view to using cytochemical markers to identify cell subpopulations, and use these markers to investigate further the changes which occur during the cellular immune response.

6. To find how M. moniliformis might survive in the cockroach haemocoel by assessing host immunocompetence.

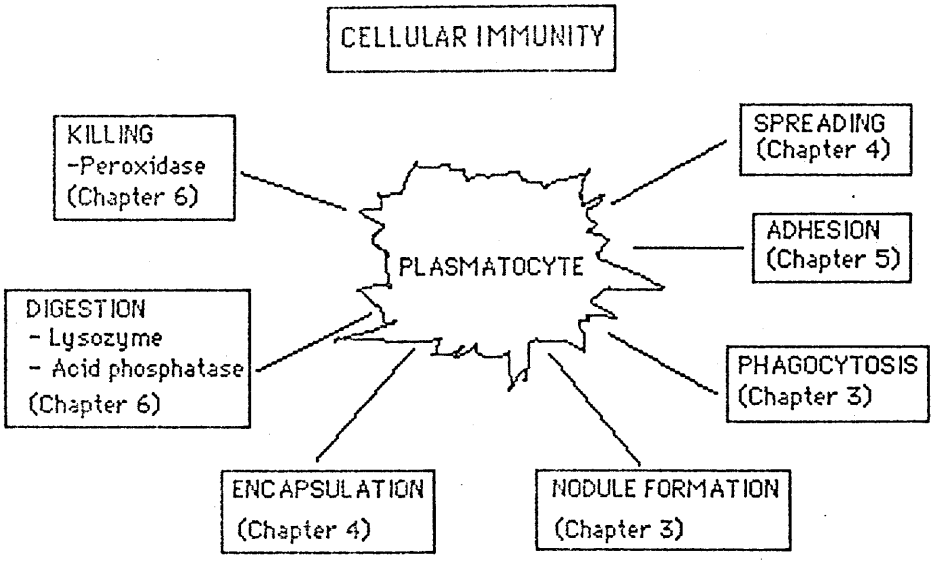


FIGURE 1.3
THE RANGE OF PLASMATOCYTE FUNCTIONS

CHAPTER 2 - MATERIALS AND METHODS

CHAPTER 2 - MATERIALS AND METHODS

2.1. GENERAL METHODS

2.1.1. ANIMAL MAINTENANCE

a. Insects

Periplaneta americana

Adult male or female P. americana were obtained from Bioserv or were reared in the insectary of the Zoology Department, Glasgow University. They were fed ratcake and water ad libitum and kept at 25°C ± 2°C on a light : dark regime of 16hrs light: 8hrs dark. Their diet was supplemented occasionally with fruit, lettuce and Bemax. They were housed in glass tanks, partly filled with peat or wood shavings, and egg cartons were provided as bedding material.

Tribolium confusum

The flour beetle, T. confusum, were raised in the departmental insectary, as above. They were housed in plastic tanks filled with wholemeal flour, over which Whatman filter papers had been laid. Occasionally, their diet of wholemeal flour was supplemented with fresh lettuce and apple.

b. Rats

Adult male and female Wistar rats were bred (random) in the animal suite of the Zoology Department. They were supplied with CRM rodent diet and water ad libitum and maintained at 21±2°C in a 12 hour light: dark regime. Prior to infection, rats were anaesthetised with ether.

c. Moniliformis moniliformis

The acanthocephalan parasite, M. moniliformis was maintained in Wistar rats and the American cockroach, P. americana.

Oral Infection of P. americana with M. moniliformis

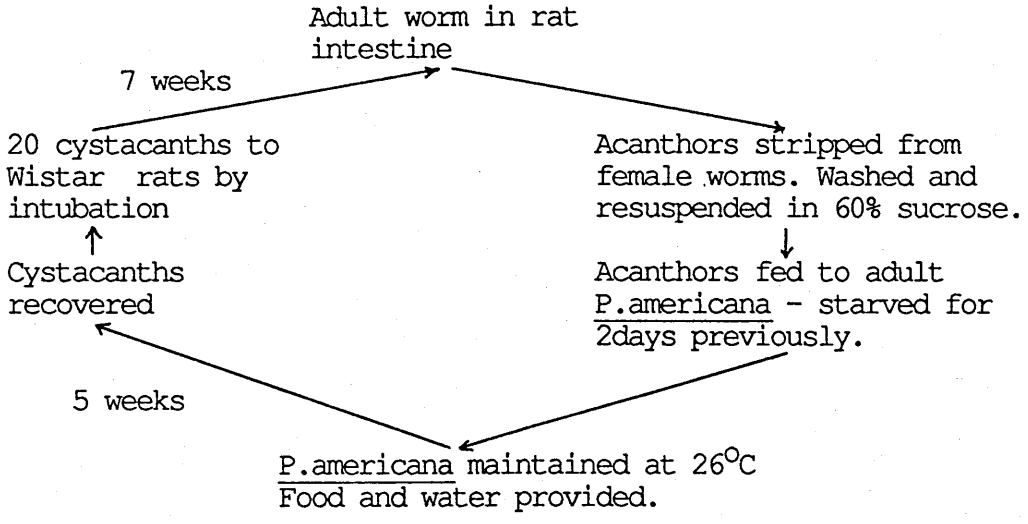
Adult P.americana were starved for 2 days, after which they were allowed to feed on shelled acanthors of M.moniliformis suspended in a 60% sucrose solution. The insects were starved for 1 day more then provided with rat pellets and water. Infected animals were maintained in the insectary at 25-27^{o}C for 5-6 weeks, after which cystacanths of M.moniliformis were recovered. CO_2 anaesthetised cockroaches were decapitated and the wings and legs removed. The abdomen was teased apart in a Petri dish containing Hepes-buffered salt (HBS) solution, pH 7.2 (Huxham and Lackie, 1986; see Appendix for recipe).

Several experiments required that the cockroaches receive a consistent dose of acanthors. In these instances, 25µl of an egg suspension was fed to the hand-held cockroaches using an Oxford pipette. Once the starved cockroaches had begun to feed, the remainder of the egg suspension was slowly expelled from the pipette. Cockroaches which either refused to feed or did not consume the complete volume of fluid were rejected. Groups of fed cockroaches were maintained in plastic containers lined with tissue and with egg cartons for bedding. Food and water was provided and the animals were returned to the insectary.

Collection of Shelled Acanthors of M. moniliformis

Adult stages of M.moniliformis were collected from the small intestine of Wistar rats, which had been infected with 20 cystacanths by intubation at least 7 weeks earlier. Adult female worms were stripped by holding the anterior end of the worm with fine forceps and cutting the posterior end of the worm. The shelled acanthors were collected into Hepes Buffered Saline (HBS) by pulling the length of the worm through forceps. The acanthors were washed 3 times by centrifugation at 250g and resuspension in HBS. The final resuspension and storage at 4°C were in 60% sucrose (J.M. Lackie, 1972); eggs can be stored in this state for several months without loss of viability.

LABORATORY MAINTENANCE OF M. MONILIFORMIS



Infection of P. americana with Acanthellae of M. moniliformis.

The method described below was used to infect cockroach hosts with a standard number of M.moniliformis larvae.

Adult and juvenile donor P.americana were orally infected with

M.moniliformis, as above, and maintained in the insectary at 26-28°C for 2-3 weeks. M.moniliformis at the acanthella I/II stage (King and Robinson, 1967) were recovered by dissection of infected cockroaches. These larvae were washed 3 times with cockroach maintenance medium (D73) (see Appendix for recipe) containing 10% v/v heat-inactivated (30min at 60°C) foetal calf serum (FCS) (Flow Laboratories, UK) to remove any adhering foreign material such as faecal waste from the dissected donor cockroaches.

Each naive recipient animal was injected with 35-40 acanthellae in 30µl D73 + 10% FCS using a sharpened hand-made glass micropipette linked to a 1ml syringe via rubber tubing. The number chosen approximates the mean number of parasites recovered from orally infected cockroaches and has been used previously (Lackie and Holt, 1988). This method permits infection of animals with a constant dose of parasites, which does not occur in orally-infected animals. Control animals received injections of medium in which larvae had been stored temporarily. Another control group received injections of medium containing portions of cockroach ovariole comparable in size to the total mass of parasites injected into experimental animals. This tissue provides a suitable control since, like the parasite, it is not encapsulated in allogeneic transplantation experiments (Lackie, 1979). Control and experimental animals were returned to the insectary and maintained under constant conditions, as described above.

After approximately 8 days infected and control animals were used in experiments on the immune response to the parasite.

INFECTION OF P. AMERICANA WITH M. MONILIFORMIS

EXPERIMENTAL PROCEDURE

Acanthors fed to adult P.americana - starved for 2 days previously.

↓

Maintain cockroaches at 26 - 28°C with food and water provided.

|

3 weeks

↓

Recover acanthellae II.

↓

Inject 35-40 acanthellae II into adult P.americana

CONTROLS - Naive
- Medium (30µl) injected into the haemocoel
- Ovariole injected into the haemocoel

d. Hymenolepis diminuta

The cestode H. diminuta was maintained in the flour beetle, T. confusum, and Wistar rats.

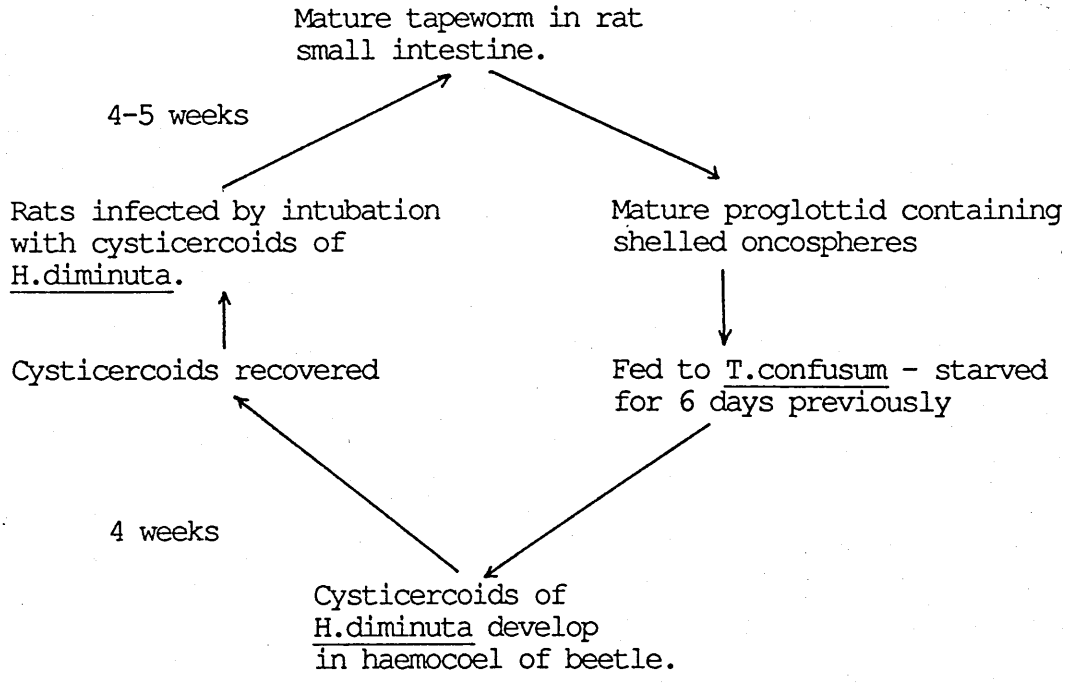
Oral Infection of T. confusum with H. diminuta

Lightly anaesthetised Wistar rats were infected with 10 cysticercoids of H.diminuta by intubation. The mature, gravid tapeworms were recovered after 4-5 weeks of development by dissection of the rat small intestine and transferred to HBS. Proglottids containing viable eggs (shelled oncospheres) were identified according to the morphological characteristics of the eggs under a dissecting microscope. Proglottids, in which the shelled oncospheres appeared rounded with the oncospheral hooks clearly present, were assumed to be mature and viable. The oncospheres in immature proglottids were similar to the

mature in size but oval in shape. Mature proglottids were teased away from the posterior end of the adult worms using fine forceps.

Adult T.confusum, which had been starved for up to 6 days, were fed on small pieces of mature proglottid soaked in HBS and placed on pieces of Whatman filter paper. The beetles were maintained in the insectary in 12cm diameter crystallising dishes at 26-28°C and fed on wholewheat flour and lettuce. After 4 weeks, the beetles were CO_2 anaesthetised and teased apart in saline under x25 magnification on a dissecting microscope to retrieve the mature cysticercoids.

LABORATORY MAINTENANCE OF H. DIMINUTA

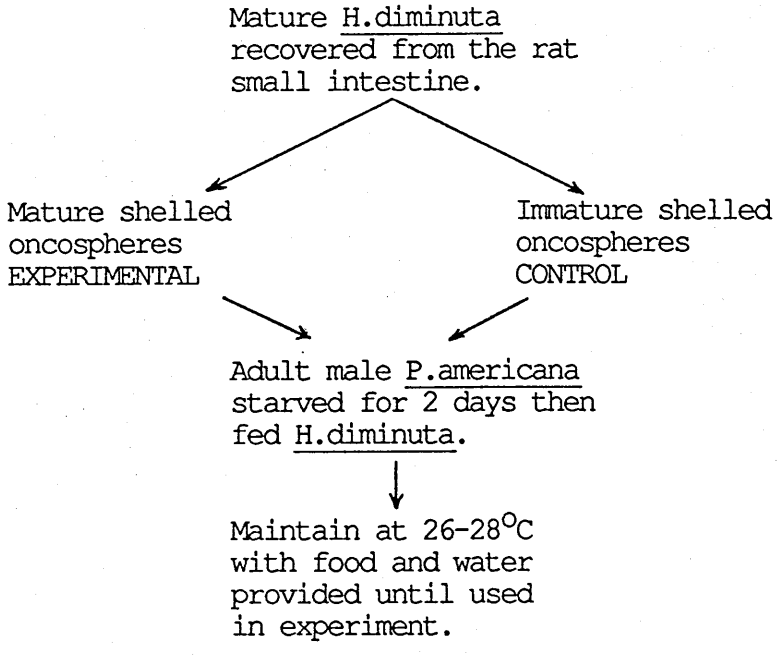


Infection of P. americana with H. diminuta

Mature and immature proglottids of H.diminuta were dipped in a 60% sucrose solution and fed to hand-held adult male P.americana, each

roach ingesting a 15-20mg portion of worm held in forceps. Cockroaches fed best when handled at around 26-28°C and held firmly between the fore-finger and thumb. After feeding, cockroaches were maintained at 28°C and provided with water ad libitum.

EXPERIMENTAL MANIPULATION WITH H. DIMINUTA



2.1.2. PREPARATION OF ZYMOSAN SUPERNATANT

Zymosan supernatant (Zs) was prepared by suspending 20mg/ml particulate Zymosan A (Sigma) in HBS and vortex mixing for 1min exactly, followed by centrifugation at 500g for 5min. Resuspension and centrifugation was repeated and the resulting supernatant centrifuged again to remove any remaining particulate zymosan.

2.1.3. SILICON COATING OF GLASS SURFACES

Glassware to be siliconised was washed with Decon detergent then rinsed with distilled water and alcohol. Each item was immersed in Sigmacote(Sigma) for 1min, then removed and allowed to dry. Finally, the siliconised glassware was cleaned with alcohol.

2.2.1. TOTAL HAEMOCYTE COUNTS(THC)

a. Cell Collection

A 10ul sample of undiluted cockroach haemolymph was collected from the stump of a severed hindleg of a CO_2 anaesthetised cockroach using an Oxford micropipette. The haemolymph was pipetted into 1ml citrate-EDTA anticoagulant buffer (pH 4.6) on ice and mixed gently and thoroughly. Total haemocyte counts (THC) were performed using a Modified Fuchs-Rosenthal haemocytometer.

b. Statistics

Except where stated, data for total haemocyte counts were compared using the unpaired Student's t-test provided by the Minitab statistics computer package. Before analysis, data were tested for a normal distribution by performing normal probability plots. The points followed the general pattern of a straight line and this was taken as evidence for a normally distributed population.

On graphs, the following convention was adopted to indicate significance:-

a = $p<0.01$
b = $p<0.05$

This was used both in this chapter and in subsequent chapters.

c. Experimental Manipulations

It is not a viable practice to sample blood sequentially from one animal before and after infection since wounding and stress must also be considered as relevant to the immune response. In these experiments, therefore, data were pooled from groups of animals to obtain information on the changes in the haemogram after treatment.

Zymosan A Supernatant Injection

The effect of Zs on the THC at various time intervals after treatment was investigated to find whether Zs could alter the THC and if so, when the effect would be greatest.

A group of 20 adult male cockroaches were each injected with 10µl Zs, prepared as described in Section 2.1.2, using a glass Hamilton microsyringe with 1µl graduations. HBS-injected (10µl) and naive animals served as controls. The animals were kept at 28°C for 30min, 1, 3 or 12hr with food and water provided ad libitum, after which a cell count was performed as above (see Section 2.2.1.a).

Infection with H.diminuta

The effect of feeding with Hymenolepis proglottids on the cockroach THC was studied since, if this cestode has a stimulatory effect on the immune system, then the number of circulating cells may increase to combat the infection.

The parasite was recovered from rats as described in Section 2.1.1.d. Groups of 20 adult, male cockroaches were fed mature proglottid and controls were fed immature proglottid. The cockroaches were kept at 28°C for 1, 3 or 5 days, after which a cell count was performed.

Infection with Acanthors of M.moniliformis

Since M. moniliformis enters the host via the gut, it was expected that the THC might change as the immune system of the host responded to either the parasite within the haemocoel or to the gut damage associated with parasite entry.

The parasite was recovered and the shelled acanthors collected as described previously (see Section 2.1.1.c). Acanthors to be fed to control cockroaches were heat-inactivated at 65°C for 30min. Groups of 20 hand-held adult male cockroaches were each fed 25ul of a suspension containing approximately $5x10^3$ live or heat-killed shelled acanthors in a 60% sucrose solution. Cell counts from control or experimental animals were carried out after 1, 3 or 5 days.

Infection with Acanthellae of Moniliformis

M. moniliformis resides in the haemocoel of the cockroaches, yet the cellular encapsulation response to these invaders is minimal. A THC was performed to find out whether the cell count is reduced in animals infected with a known dose of parasites. Forty acanthellae II were injected into each of 20 adult, female P. americana (see Section 2.1.1.c). Two control groups (20 animals each) received either portions of ovariole or medium injections. The animals were kept at 28°C for 8 days, after which THCs were performed, as above. Infected cockroaches were dissected and the number and stage of the parasites was noted.

2.2.2. PHAGOCYTOSIS

a. Cell Preparation

Control and experimental animals were CO_2 anaesthetised and injected with 10µl 2% FITC-latex spheres (0.21µm diameter) in HBS using a Hamilton microsyringe. Animals were incubated at 28°C after which cold HBS (200µl) was injected into the abdominal haemocoel of CO_2 anaesthetised animals, using a 1.0ml plastic syringe and a 25 gauge hypodermic needle. Haemolymph that exuded from the coxa of a severed hindleg was collected, using a pasteur pipette, into 1ml HBS on ice. A few drops of the diluted haemolymph were pipetted onto a clean glass slide and haemocytes, allowed to settle and spread at room temperature for 30min, were then fixed using 2% paraformaldehyde in HBS. Slides were mounted in 50% (v/v) glycerol/phosphate buffered saline mixture.

b. Cell Counting

Fixed haemocytes were observed initially using a x25 objective and phase contrast optics on a Leitz Ortholux II microscope and categorised according to cell type ie. plasmatocyte or coagulocyte to obtain information on the proportion of each cell type in one field of view. Each field was also examined using incident light fluorescence with an HBO 50 high pressure mercury vapour lamp. For the FITC reactions, 2X KP490 (exciting), TK 510 (dichroic mirror) and K515 (suppressing) filters were used. Cells were classified on intensity of staining such that, for example, for phagocytosis a cell with fewer than 25 beads internalised was designated as having a low phagocytic uptake whereas a strongly fluorescent cell had a high phagocytic

uptake. The difference between the total number of each cell type counted under phase contrast (200-300 cells counted/animal) and the total for each cell type containing fluorescent material provided information on the number of negative cells. The percentage of plasmatocytes containing fluorescent beads according to the scoring regime, was calculated for each animal and a mean percentage and standard deviations for each group were calculated.

c. Statistics and Graphical Representation

Percentage data were arcsine transformed before analysis using oneway analysis of variance (ANOVA). Each replicate was analysed in this way using the Minitab computerised statistics package.

The variance ratios for each treatment in individual experiments were compared to find whether results could be grouped to obtain larger sample sizes. Since the experiments were performed in an identical manner and the variance ratios were not significantly different, it was concluded that this was a valid procedure. However, where appropriate the results for individual experiments will be quoted.

For simplicity, the data for the weakly and intensely phagocytic cells are presented as stacked columns. The height of each column on the graph represents the total percentage of plasmatocytes which were phagocytic, composed of the strongly phagocytic cells stacked on the weakly phagocytic cells. Significance values quoted on the figures represent comparisons within experiments of the total phagocytic haemocytes.

d. Experimental Manipulations

Time Course for Phagocytosis

The THC and the number of phagocytic cells at various times was investigated in order to establish the dynamics of the cell population after injection with an abiotic particulate material (latex spheres). Adult P.americana were injected with 10µl 2% FITC-latex spheres and haemolymph was collected either 15 min, 30 min, 1hr or at hourly intervals thereafter, up to 8 hr after injection. Each group contained 20 adult female cockroaches.

The THC after latex-sphere or saline injection was counted (see Section 2.2.1.) before the haemolymph was sampled for the phagocytosis assay. Immediately after collection of undiluted haemolymph for the THC, the animals were injected with HBS and diluted haemolymph was collected into HBS. The number of cells which had phagocytosed spheres at each time interval was determined as above.

Phagocytosis After Zymosan Supernatant Injection

Adult male P. americana were CO_2 anaesthetised and injected with either 10µl 2% Zs or HBS. Naive animals provided a further control. Following treatment, cockroaches were maintained for 12hr at 28°C and provided with food and water ad libitum. For assaying phagocytosis, experimental and control animals received a further injection of 10µl 2% FITC-latex beads in saline. After 2hr at 28°C, the haemolymph was collected and the phagocytic cells counted as above.

This experiment was repeated with the animals receiving the latex spheres 3hr after the Zs injection. The haemolymph was collected 2hr later.

Phagocytosis After Infection with H. diminuta

The phagocytic uptake of FITC-latex spheres by haemocytes of experimental and control animals was investigated at 1, 3, 5 and 8 days after ingestion of mature or immature Hymenolepis proglottids (see Section 2.1.1). FITC-labelled latex spheres were injected into the haemocoel of each animal, as above, and after incubation for 2hr at 28°C, the haemolymph was collected and the number of cells which had phagocytosed spheres was determined.

2.2.3. NODULE ASSAY

The nodule assay serves as a useful indicator of immune responsiveness (Gunnarsson and Lackie, 1985) and is used as such in this work.

a. Cell Preparation

The number of cell aggregates or nodules present in the haemolymph of adult male P. americana was determined by flushing out the haemolymph with approximately 1ml Hanks calcium and magnesium-free saline containing 5% EDTA (CMF-EDTA), an anticoagulant solution which prevents coagulation of individual cells but maintains nodules intact (Gunnarsson and Lackie, 1985). Diluted haemolymph exuded from the stumps of 3 severed legs was collected into 1-2ml Hanks CMF-EDTA in a 35mm Nunc plastic culture dish and agitated gently.

The nodules were counted on a Leitz Ortholux microscope using x40 magnification and phase contrast optics.

b. Statistics

Data were analysed using the unpaired two-way Student's t-test on the

Minitab package.

c. Experimental Manipulations

Nodule Production After Zymosan Supernatant Injection

The stimulatory effect of Zs on nodule production 12hr after treatment was investigated. Two groups each of 8 adult male cockroaches were injected with 10µl of Zs (see Section 2.1.2.). HBS-injected and naive animals served as controls. The animals were kept at 28°C for 12hrs. The nodules were collected from one of the Zs treated groups. The remaining Zs treated group and the control groups received a second injection of 10ul HBS. The nodules were collected from these groups 3hr later.

The above experiment was repeated with cockroaches receiving the second injection only 3hr after the initial Zs injection.

SECTION 2.3. ENCAPSULATION AND SPREADING

2.3.1. IN VITRO ENCAPSULATION OF SEPHAROSE BEADS

a. Preparation of Sepharose Beads

Sepharose beads were used in an in vitro encapsulation assay to investigate cellular adhesion to substrata of different charge and cellular adhesion after manipulation of the immune system.

Before use, the beads were washed extensively in HBS to remove the storage fixative. A 1ml volume each of Sepharose-CL-6B (neutral charge), diethyl amino ethyl (DEAE)-Sepharose-CL-6B (positive charge) and carboxy methyl (CM)-Sepharose-CL-6B (negative charge) was suspended separately in 20-30ml HBS (pH7.2) and centrifuged at 400g for 5min, the

supernatant removed and the pellet resuspended in HBS. Centrifugation and resuspension were repeated twice. Washed beads were passed through a 100µm mesh Nitex filter and those retained by the filter were used in the experiment. Using a modified Fuchs-Rosenthal haemocytometer, a count was performed and the beads were resuspended to a concentration of $2x10^3$ beads/ml HBS.

b. Preparation of Cockroach Haemocytes

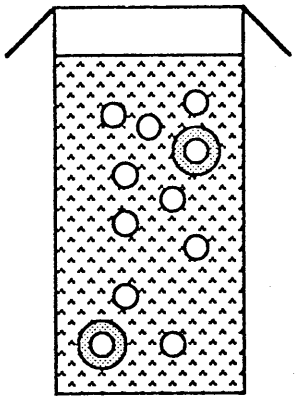
Adult female P. americana received intra-haemocoelic injections of 200ul citEDTA anticoagulant using a 1.0ml plastic syringe with a 25 gauge hypodermic needle. Haemolymph which exuded from the coxa of a severed hindleg was collected with a pasteur pipette, pooled into citEDTA and centrifuged at 250g and room temperature for 5min. After another centrifugation, haemocytes were counted using a modified Fuchs-Rosenthal haemocytometer and resuspended in a small volume (500µl) of HBS at 4°C to which D73+10% FCS was added. The final cell concentration was $0.5x10^6$ haemocytes/ml D73 cockroach haemocyte maintenance medium.

c. Preparation of Encapsulation Vials

Encapsulation vials were siliconised (Sigmacote, Sigma) glass test-tubes (500ul volume) sealed at the top with Nescofilm (see Fig 2.3.1., after Davies and Vinson, 1986). Haemocytes were added to encapsulation vials containing Sepharose beads to a final ratio of 250 cells : 1 bead. A small air bubble was left in the encapsulation vial which was then sealed with Parafilm and agitated thoroughly by hand to mix the cells. This avoided coagulation of the cells and sticking of the target to the walls of the vessel. The vials were rotated gently at room

temperature using an end-over-end mixer (Emscope) with small clips to hold the encapsulation vials. At each rotation, the air bubble tumbled the contents of the vial. After 1hr, the contents of the vials were fixed with either 4% formaldehyde in HBS for light microscope examination and acid phosphatase visualisation or 2.5% glutaraldehyde for electron microscope examination and peroxidase visualisation.

FIGURE 2.3.1. - THE IN VITRO ENCAPSULATION VIAL



Glass encapsulation vial sealed with Parafilm leaving small air bubble for mixing of cells.

Dimensions :-

Length = 25mm

Diameter = 5mm

Haemocytes in D73 cockroach medium

Unencapsulated Sepharose bead target

Encapsulated Sepharose bead target

d. Preparation of Encapsulated Sepharose Beads for Electron Microscope Examination

Encapsulated beads were fixed with 2.5% glutaraldehyde for 1hr, rinsed with 0.1M cacodylate buffer then postfixed in 1% aqueous osmium tetroxide and stained with 0.5% aqueous uranyl acetate. After dehydration in an alcohol concentration series, the specimens were blocked in Araldite and thin sections were cut using an LKB ultramicrotome. Sections retained on grids were stained with uranyl acetate (0.5%) and lead citrate(0.5%).

e. Experimental Manipulations

Cell Number and Duration of Culture

The cell number included in the vial and the culture time were varied to find the optimum conditions for encapsulation. The cell densities used were 0.25, 0.5, 1 and $2x10^6$ cells/ml medium with a 1hr incubation period. The experiment was repeated three times with pooled cells from 10 naive adult male cockroaches.

A cell suspension of $0.5x10^6$ haemocytes/ml medium was cultured for 3hr with the target particles. Samples were collected and fixed (4% formaldehyde in HBS) at hourly intervals and the extent and structure of the capsule examined using phase contrast optics.

Haemocytes were tested for viability at the initial stages of the experiment and after several hours in culture. Samples (30µl) were collected from the culture vial and incubated for 10min at room temperature with 0.1% trypan blue in D73+10%FCS. After thorough washing, haemocytes were examined for the presence of blue dye within the cytoplasm which indicates cell death.

The Characteristics of Encapsulating Haemocytes

Haemocytes were prepared as in Section 2.3.1.b above. After incubation for 1hr with the target particles, haemocytes were separated into 2 samples and fixed for 30min at room temperature with either 4% formaldehyde in HBS (acid phosphatase) or 2.5% gluteraldehyde in HBS (peroxidase). After thorough washing in HBS, samples were processed for enzyme visualisation (see Section 2.5 for Materials and Methods). Blue deposits indicated the acid phosphatase activity, whereas dark brown product was produced by the peroxidase reaction.

To determine whether the phagocytic cell population might be involved in capsule formation, cockroaches were preinjected with particulate material (10µl 2% FITC-latex spheres in HBS; see Section 2.2.2.a for detailed method) and haemocytes were used 2hr later in the encapsulation assay. The cells were incubated for 1hr with the beads, then fixed (4% formaldehyde in HBS) for 30min at room temperature and examined for fluorescent beads within the cells forming the capsules.

Infection with H. diminuta

Cockroaches were fed H.diminuta and maintained for either 1 or 3 days, as in Section 2.1.1.d. Haemocytes were collected from these animals and used in the in vitro encapsulation assay. Cell and Sepharose bead preparation were as described above. Encapsulation vials were set up as follows:-

4 vials = cells (naive animals) + DEAE-Sepharose
4 vials = cells (immature Hymenolepis) + DEAE-Sepharose
4 vials = cells (mature Hymenolepis) + DEAE-Sepharose

The number and morphology of adherent cells was examined with phase contrast optics using a x20 (long working distance) objective on a

Leitz Diavert inverted microscope.

2.3.2. *IN VIVO* ENCAPSULATION OF SEPHAROSE BEADS

a. Preparation of Sepharose Beads

Sepharose beads were used in an *in vivo* encapsulation assay (Lackie, 1983) to investigate the effect of parasitism on haemocyte adhesion.

Negatively charged (CM-) Sepharose beads were used in the *in vivo* encapsulation assay. Preparation of the beads was performed as for the *in vitro* encapsulation assay. The beads were resuspended in cockroach haemocyte maintenance medium (D73) containing 10% (v/v) foetal calf serum (FCS) to a concentration of 1.5×10^3 beads/ml.

b. Statistics

For simplicity, data from individual animals were amalgamated for each treatment and, assuming the animals were from the same population, the means were analysed.

Since data on capsule thickness was found to be not normally distributed using normal probability plots. Non-parametric tests, the Mann-Whitney and the Kruskal-Wallis rank-sum tests, (Minitab computerised statistical package) were used for statistical analysis. The following convention was used to indicate levels of significance:-

a = $p<0.01$
b = $p<0.05$

c. Experimental Manipulations

Infection with *H. diminuta*

Adult, male *P.americana* were fed either mature or immature *H.diminuta*

proglottids as in Section 2.1.1.d. Cockroaches were maintained for either 1 or 3 days at 26-28°C with food and water provided. Each group contained 8 animals.

Using a sharpened glass micropipette, cockroaches received 30ul intra-haemocoelic injections containing approximately 40 CM-Sepharose-CL-6B beads (negatively charged) and the abdomen was palpated several times to disperse the beads. Encapsulated and unencapsulated Sepharose beads were recovered after 24hr at 28°C and fixed in 2.5% gluteraldehyde in HBS. The capsule thickness was measured at 4 positions around the bead using an eyepiece graticule, calibrated with a stage micrometer, and phase contrast optics on a Leitz microscope. A mean value of capsule thickness for each bead was calculated.

Infection with Acanthellae of M. moniliformis

Adult, female P.americana were infected with 40 acanthellae II of M.moniliformis as in Section 2.1.1.c. Control animals received either 30ul medium or portions of ovariole in medium. After 8 days at 28°C, with food and water provided, cockroaches received injections (30µl) of approximately 40 CM-Sepharose-CL-6B beads (Section 2.3.1.). Encapsulated beads were recovered 24hr later and fixed with 2.5% glutaraldehyde in HBS. The capsule thickness was measured as above for H.diminuta

2.3.3. HAEMOCYTE SPREADING

a. Preparation of Substrata

Coverslips of 13mm diameter (Chance Propper Ltd.) were washed with Decon 90, rinsed thoroughly in distilled water and cleaned with absolute alcohol. They were rendered pyrogen-free by incubation at

160°C for 3hr then placed in Sterilin multiwell plates and incubated at room temperature for 30min with the protein to be tested (250µl of either 0.1, 1 or 10 mg/ml BSA in HBS or 10% (v/v) FCS in HBS). Clean glass coverslips served as controls. After coating, the protein was removed from the wells and the coverslips rinsed with HBS.

b. Preparation of Cockroach Haemocytes

Haemocytes were collected from adult male P. americana by injection of HBS (200µl) into the haemocoel and dilution in HBS (1ml) of haemolymph which exuded from a hindleg stump. Haemocytes (25µl in HBS) were pipetted onto the coverslips (see below) and incubated for 30min at 24-25°C after which the excess fluid was removed and replaced with 10% (v/v) formaldehyde in HBS. Fixed haemocytes (30min) were stained for 30min with 0.1% Kenacid blue in stain solvent (water: methanol: acetic acid, 50:50:7 by volume) then rinsed twice with distilled water and air-dried.

c. Analysis of Spread Area

The spread area of haemocytes was measured using the method of Edwards et al. (1987) for fibroblast cells. Images were obtained using a 20x objective on a Leitz Diavert microscope linked to a video camera. The images were digitised for input to the screen memory of a BBC microcomputer using a Data Harvest Video Interface. The captured images were displayed on-screen in three shades excluding the background shading. The spread area of at least 100 cells on each coverslip were measured by counting the total connected pixels for each cell using specially written software.

c. Statistics and Graphical Representation

The spread areas of cells are expressed as a percentage of that on clean glass, assuming that the cells spread completely on clean glass. By expressing the results as a proportion of spread area on glass, each sample is normalised and external variation due to enviromental factors, such as temperature variations is minimised.

Haemocyte spread areas were compared using non-parametric methods, the Mann-Whitney rank-sum test, and significance levels were indicated as in Section 2.3.2.b, above.

d. Experimental Manipulations

Bovine Serum Albumin and Foetal Calf Serum

In these preliminary experiments, spreading of haemocytes on different proteins was studied to find a surface to which haemocytes would stick without spreading completely. Such a protein was to be used in later experiments so that enhancement or inhibition of spreading could be measured.

Haemocyte spreading on coverslips coated with BSA (0.1, 1 and 10mg/ml) or FCS (10% in HBS) were compared using cells from naive cockroaches.

Zymosan Supernatant Injection

Adhesion of haemocytes is influenced by Zs (Gunnarsson and Lackie, 1985) and since adhesion and spreading are often associated processes, the effect of Zs on the spreading of haemocytes was investigated.

Haemocytes were collected from cockroaches injected with Zs 3hr before and the cell spread areas after 30min incubation on BSA-coated

glass coverslips (1mg/ml BSA in HBS) were measured, as above.

Infection with H. diminuta

Oncospheres of H. diminuta become encapsulated by a multilayered sheath of cockroach haemocytes in which cells spread extensively. In this experiment, the spreading of haemocytes from infected animals was investigated to find whether an enhanced ability to spread is indicative of cellular activation.

Spreading on BSA-coated glass (1mg/ml BSA in HBS) by haemocytes from cockroaches fed Hymenolepis 1day earlier was compared with naive animals or animals fed with non-viable Hymenolepis proglottids.

SECTION 2.4. ADHESION IN THE FLOW CHAMBER

2.4.1. Cell Preparation

Haemocytes from 10 adult male P.americana were collected with citEDTA anticoagulant (pH 4.6) and pooled, as described in Section 2.3.1.b.. A cell count was performed using a modified Fuchs-Rosenthal haemocytometer. Several aliquots of known cell number were removed and washed once more in citEDTA. Before use, cells were resuspended in HBS to a concentration of 0.5×10^6/ml.

Since haemocytes, especially coagulocytes, lyse readily in vitro a limited number of experiments could be done on a single cell preparation. It was decided, therefore, to use a cell preparation for only two flow chamber assays. Thus, variability arising from lysis because of an extended time in anticoagulant was avoided.

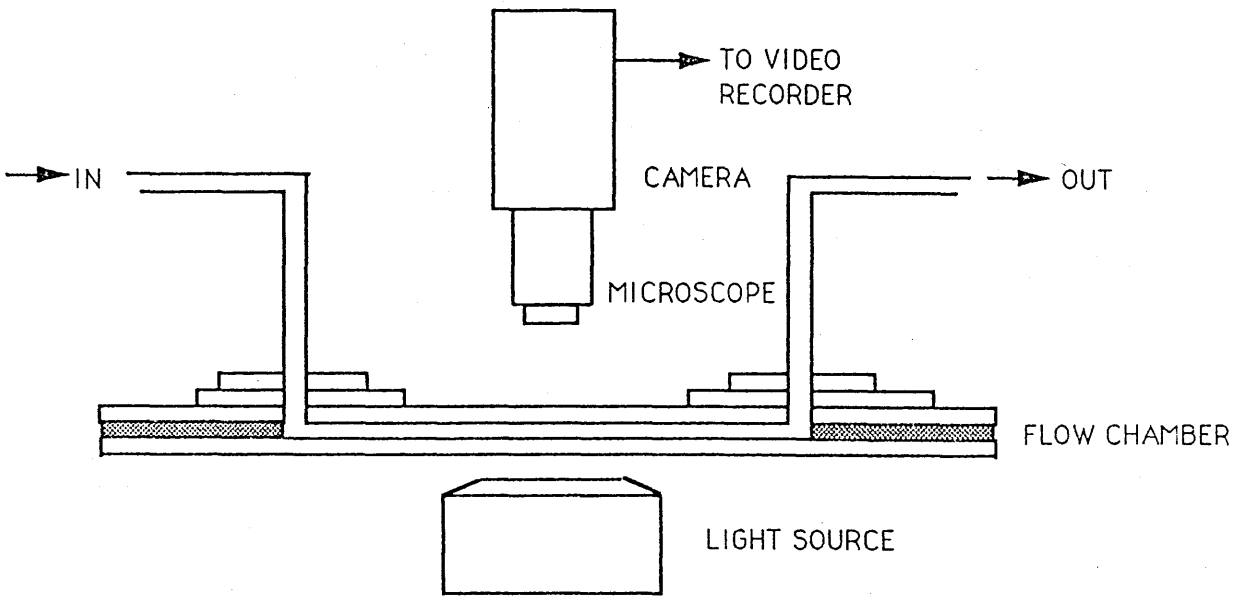


FIGURE 2.4.2.a - THE FLOW CHAMBER
Lateral view of the assembled flow chamber.

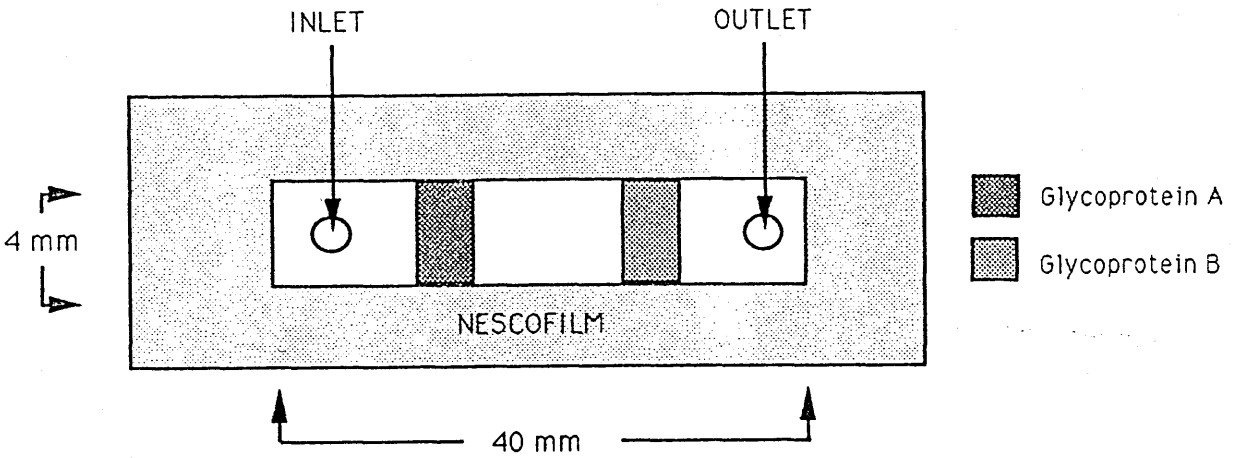


FIGURE 2.4.2.b
Diagrammatic plan view of the flow chamber showing the position of the glycoprotein stripes which are dried onto the glass before the chamber is assembled with Nescofilm.

2.4.2. Flow Chamber Preparation

The flow chamber preparation was as described in Forrester and Lackie (1984). A flow chamber was constructed between two microscope slides separated by a strip of Nescofilm in which a channel (40mm x 4mm) had been cut (see Fig. 2.4.2.b). The upper slide had 4mm holes drilled through it and these coincide with inlet and outlet ports of a metal and perspex clamping device.

Each assembled flow chamber (see Fig. 2.4.2.a) was perfused with 10% (w/v) bovine serum albumin (BSA) 10min before use to coat the lower glass slide with protein and avoid non-specific adhesions to the glass surface. Haemocyte suspensions were introduced into the chamber at a constant flow rate (8ml/hr) using a mechanical syringe drive. Cells adhering to the lower surface of the chamber were viewed using a x20 objective on an inverted Leitz microscope and a monochrome video-camera/monitor system in conjunction with a time lapse video recorder with a time-date generator. The temperature of the chamber (28^{o}C) was regulated by a fan which circulated warm air over the microscope stage. Cells were perfused through the chamber for 5min exactly. Each chamber was washed with HBS for 3-5min (flow rate = 18ml/hr) to remove non-adherent cells and the remaining adherent haemocytes were fixed with 4% formaldehyde in HBS.

2.4.3. Cell Counting

Random counts of cells sticking to the different surfaces were performed using a x20 objective on a Leitz inverted microscope and phase contrast optics. The number of cells in eight randomly chosen areas of a field were counted and 6 fields were examined for each

surface, either BSA or glycoprotein-coated strips.

2.4.4. Statistics

The total number of cells counted on each coating are expressed as a percentage of the cells adhering to the first BSA-coated glass area which was normalised to 100%. Handling the data in this way reduced variation between experiments which simplified interpretation of the results and graphical representation. Percentage data were compared using the Mann Whitney two sample rank test provided on the Minitab computerised statistics package.

Values of significance were denoted as a=$p<0.01$ and b=$p<0.05$.

2.4.5. Experimental Manipulations

Adhesion to Asialo vs Native PSM in Naive Cockroaches

Two 0.5cm wide strips of asialo porcine stomach mucin (PSM) and native PSM (1mg/ml in HBS) were air-dried onto the lower slide (see Fig.2.4.2.b). Haemocytes were collected from naive, adult male P. americana using citEDTA as described previously (Section 2.4.1). Each cell preparation was used for two flow chambers, such that either asialo or native PSM was encountered first by the cells.

Zymosan Supernatant Injection

Ten CO_2-anaesthetised adult male P.americana were each injected with 10µl 2% Zymosan A (Sigma) supernatant, prepared as described in Section 2.1.2. Control groups were either naive or injected with 10µl HBS. The cockroaches were incubated for either 1hr or 3hr at 28°C. Haemocytes were prepared for adhesion studies as in Section 2.4.1.

Strips (0.5cm wide) of porcine stomach mucin (PSM) and bovine submaxillary mucin(BSM) (1mg/ml in HBS) were air-dried onto Decon washed slides (see Fig. 2.4.2.b). PSM (Sigma) contains 1% bound sialic acids whereas BSM (Sigma) contains approximately 5% bound sialic acids. Flow chamber construction and cell counting were as in Section 2.4.2./3

Infection with H. diminuta

Adult, male P.americana (8 animals/group) were fed either immature or mature proglottids of H.diminuta (see Section 2.1.1). Haemolymph was collected at either 1 or 3 days after feeding. The number of haemocytes adhering to either protein (BSA)-coated glass or glycoprotein (BSM and PSM)-coated glass was determined for H.diminuta-fed animals and compared to controls.

This experiment was repeated with cells adhering to either native or asialo-BSM on the lower slide.

Infection with Acanthors and Acanthellae of M. moniliformis

The number of haemocytes adhering to either protein (BSA)-coated glass or glycoprotein (BSM and PSM)-coated glass under conditions of flow was determined for cockroaches infected with acanthors or acanthellae of M.moniliformis.

Haemolymph was collected (see Section 2.4.1.) from adult female cockroaches, which had been infected with acanthellae II of M.moniliformis 8 days previously (see Section 2.1.1.c). Controls were either injected with medium only or with medium containing portions of ovariole.

Adhesion by haemocytes to the glycoproteins was also investigated for

cockroaches fed live or heat-inactivated acanthors (30min at 65°C) 2 days before experimentation.

SECTION 2.5 CYTOCHEMISTRY

2.5.1. Cell Preparation

Acid Phosphatase and Peroxidase

HBS (200µl) was injected using a plastic syringe and 25 gauge needle into CO_2 anaesthetised male P. americana and haemolymph exuded from the base of a severed hindleg was collected with a pasteur pipette into 1ml HBS on ice. The diluted haemolymph was pipetted onto a clean glass slide and haemocytes were allowed to spread at room temperature for 30min after which they were fixed for 30min in 4% formaldehyde for acid phosphatase staining, or 2.5% glutaraldehyde in HBS for peroxidase staining.

Lysozyme

To avoid the problem of non-specific adsorption of reagents to serum components, cells were collected in anticoagulant and washed. By this method, serum proteins are removed and cellular lysozyme, rather than serum lysozyme, can be more accurately determined. Haemolymph from adult, male P. americana was collected with citEDTA, pooled into citEDTA and washed twice by centrifugation at 250g for 5min and resuspension in citEDTA. The cells were resuspended finally in HBS on ice. Pooled haemolymph (15µl) was pipetted onto each well of a 12 well slide. Three replicate slides were prepared from each cell preparation. The cells were allowed to adhere and spread for 30min

then fixed with 2% (w/v) paraformaldehyde in HBS for 30min.

2.5.2. Enzyme Localisation

Acid Phosphatase

Sites of acid phosphatase activity were localized using the azo-dye coupling method, based on that of Burnstone (Pearse, 1968). Fixed cells were incubated for 60min in a 0.1M sodium acetate-acetic acid buffer (pH 5.0) containing 0.02% Naphthol-AS-BI phosphate as substrate, 0.06% Fast Blue BB as dye and 5µl DMSO/ml buffer. Control slides were incubated for 1hr at 28°C in either the incubation medium containing 0.2M sodium fluoride as inhibitor or with the substrate omitted. After rinsing thoroughly with buffer, slides were mounted in Aquamount (Gurr).

Peroxidase

Sites of peroxidase activity were localized using 3,3'-diaminobenzidine-tetra-HCL (DAB) as substrate, according to a method introduced by Graham and Karnovsky (1966). Cells were incubated in a 0.05M Tris-HCL buffer(pH 7.6), containing 0.5mg DAB/ml and 10ul concentrated H_2O_2/ml. Control slides were incubated in either the incubation solution containing 0.02M aminotriazole to inhibit catalase or in the absence of either the DAB or H_2O_2 . Slides were mounted in Aquamount.

Lysozyme

All slides were incubated for 60min with 3% (w/v) bovine serum albumin (BSA) in HBS in order to block non-specific binding of antibody to

free aldehyde groups. Cell membranes were permeabilised by Triton X-100 treatment (0.5% (v/v) in HBS containing 0.1% (w/v) BSA) for 5min at room temperature followed by 2x15min washes in 0.1% BSA in HBS. Primary antibodies were diluted 1:200 with 0.5% BSA in HBS, found by titration to be a suitable concentration for enzyme visualisation.

Each slide contained 8 experimental wells and 3 control wells. Rabbit anti-Cecropia lysozyme antibody was added to each of the experimental wells. Negative controls either lacked primary antibody or were incubated with rabbit anti-Cecropia P4 antibody, an antibody prepared against the antibacterial protein (P4) of Lepidoptera, shown to provide negative results when incubated with P.americana haemocytes. A positive control was supplied by S.gregaria haemocytes incubated with anti-lysozyme primary antibody. All antibodies were kindly supplied by Dr. T. Trenczek.

Slides were incubated overnight at 4^{o} C in a humid chamber, then washed for 1hr in 0.1% BSA in HBS with four saline changes. A fluorescein isothiocyanate (FITC)-conjugated goat antibody specific for rabbit immunoglobulin (IgG) was diluted 1:100 with 0.1% BSA in HBS and added to each well of all slides. A 2hr incubation in a humid chamber at room temperature and in darkness was followed by a 1hr rinse with HBS to remove non-specific staining with the second antibody.

Slides were mounted in 50% (v/v) glycerol/phosphate buffered saline mixture and examined with a Leitz Ortholux II microscope using incident light fluorescence with an HBO 50 high pressure mercury vapour lamp. For the FITC reactions, 2X KP490 (exciting), TK 510 (dichroic

mirror) and K515 (suppressing) filters were used.

2.5.3. Cell Counting

All observations were made using a x25 objective and a CB12 filter on a Leitz Ortholux microscope. Haemocytes fixed in monolayer were observed initially using phase contrast optics and categorised according to cell type ie. plasmatocyte or coagulocyte to obtain information on the proportion of each cell type in one field of view. Each field was also examined either with epifluorescence, for the lysozyme staining, or with bright field optics for the acid phosphatase and peroxidase cytochemical assays. Cells were classified on intensity of staining. The difference between the total number of each cell type counted under phase contrast (200-300 cells counted/animal) and the total for each cell type containing stained material provided information on the number of negative cells. The percentage of plasmatocytes containing cytochemical stain, according to the scoring regime, was calculated for each animal and a mean percentage and standard deviation for each group was calculated.

2.5.4. Statistics

Percentage data were arcsine transformed then each replicate was analysed using oneway analysis of variance (ANOVA) on the Minitab computer statistics package. Data from replicate experiments were grouped to obtain larger sample sizes. However, where appropriate the results for individual experiments will be quoted. For simplicity, the data for the weakly and intensely stained cells are presented as stacked columns.

2.5.5. Experimental Manipulations

Zymosan Supernatant Injection

Zs was prepared as described previously (see Section 2.1.2.). Eight adult, male P. americana were CO_2 anaesthetised and injected, using a Hamilton microsyringe, with 10µl 2% Zs or HBS. Naive animals provided a control. Following treatment, animals were maintained for either 3hr or 12hr at 28°C and provided with food and water ad libitum.

Haemocytes were collected, fixed and processed for acid phosphatase, peroxidase as above. The experiment was repeated three times.

Infection with H.diminuta

Adult, male P. americana were fed either mature or immature proglottids of the cestode H.diminuta, as in Section 2.1.1. Cockroaches were maintained at 28°C for 24hr and fed on rat pellet and water. Haemolymph was collected into HBS (Section 2.5.1.) and the haemocytes were stained for acid phosphatase and peroxidase (Section 2.5.2.). The number of cells staining for each enzyme and the intensity of stain was studied.

The above experiment was repeated 3 days after the cockroaches had fed on H.diminuta.

Infection with Acanthellae of M. moniliformis

Adult, male P. americana were infected with 35-40 acanthellae II of M.moniliformis, as described in Section 2.1.1.c. Portions of ovariole in D73+10% FCS injected into the haemocoel of 8 animals served as a

control for the presence of M.moniliformis within the haemocoel. Another control group received 30ul injections of D73+10% FCS. After treatment, the cockroaches were returned to the insectary and maintained at 28°C for 8 days.

Cell collection, enzyme visualisation (acid phosphatase and peroxidase) and counting were carried out as above.

Phagocytosis and Superoxide Production

To determine whether phagocytic uptake of particulate material resulted in superoxide formation, a nitroblue tetrazolium (NBT) reduction assay was used. After collection with HBS (see Section 2.5.1) haemocytes were allowed to adhere to multiwell glass slides in a moist chamber at room temperature for 30min. After adhesion, the haemocytes were rinsed in HBS and 50ul NBT solution (3mg/ml HBS) was added to each well. A 10ul suspension of Zymosan A particles was added to the wells. Different concentrations of particles were used ($1.5x10^5$ - $1.5x10^7$) to obtain an optimal phagocytic uptake by the cells. Slides were incubated at 28°C for 30, 60 or 120mins then fixed with 10% formaldehyde in HBS and mounted with glycerol in PBS (1:1 v/v). Slides were studied for dark blue formazan deposits indicative of superoxide production.

In control experiments, NBT or Zymosan A particles were omitted. Superoxide dismutase (50µl 5mg/ml HBS) was preincubated with haemocytes to investigate the effects of this superoxide inhibitor on formazan production. Each experiment was repeated three times with haemolymph collected from individual male P. americana.

CHAPTER 3 - CELL COUNTS, PHAGOCYTOSIS AND NODULE FORMATION

CHAPTER 3 - THC, PHAGOCYTOSIS AND NODULE FORMATION

INTRODUCTION

This chapter introduces the haemocyte types present in adult cockroaches, the morphology of the cells, the relative proportions of phagocytic cells and the total number of haemocytes in circulation in the stimulated and unstimulated immune system.

Cell Number - An indicator of Immune Status?

Although perhaps a crude indicator of immune status, the total circulating number in naive and infected animals is essential background information to interpret the dynamics of the immune response described in later chapters.

In an immune system which depends largely upon cellular components for surveillance, the absolute cell number typical of a species or an individual of that species may be expected to determine efficacy. Thus, a large number of haemocytes would imply the capacity to produce dense cellular capsules and nodules around foreign material and a high potential for bacterial clearance by phagocytosis. Similarly, a low cell number may indicate difficulty in mounting an appropriate immune response and therefore, susceptibility to infection.

The total cell count varies throughout the life of the animal, being influenced by either endogenous factors, such as age, or exogenous factors, such as infection. The sex (Lackie et al., 1985), age (Dunphy and Nolan, 1980a; Shapiro, 1979) and nutritional status (Steinhaus and Dineen, 1960; Shapiro, 1967) of insects are all known to influence the haemocyte number.

Variations in the absolute number may occur in response to

alterations in the cell adhesiveness, such that circulating haemocytes marginate to the haemocoelic linings or blood cells are recruited from marginated reservoirs into the circulating cell pool. Thus the total cell number will be maintained as a dynamic balance between the marginated and the circulating pool. Fluctuations in the blood cell number may also occur if cells die or if existing cells undergo mitosis. Since mitosis is rarely observed in circulating insect haemocytes (Ryan and Nicholas, 1972), division of these cells is unlikely to have a profound effect on the cell count. However, Christensen et al. (1989) have recently reported that increased haemocyte populations in microfilariae-infected mosquitoes probably arise from mitotic division of circulating cells. Finally, haemopoietic tissue may release sessile and immature blood cells.

The factors discussed above can be referred to as intrinsic factors and maintain the cell number in the naive animal. However, the effects of extrinsic factors, such as wounding or infection, are superimposed on the basic cell count. Many authors have reported alterations, both quantitative and qualitative, in the insect haemogram after injury or saline injection (Guzo and Stoltz, 1987; Dunphy et al., 1986; Dunphy and Webster, 1985; Gunnarsson, 1987) and infection with bacteria (Ryan and Nicholas, 1972; Hoffman et al., 1974; Dunphy and Webster, 1984), fungi (Gunnarsson, 1987), protozoa (Ibrahim et al., 1986), helminths (Dunphy and Webster, 1985, 1988; Andreadis and Hall, 1976; Nappi and Christensen, 1986) and parasitoids (Nappi, 1981; Guzo and Stoltz, 1987; Davies, Strand and Vinson, 1987).

Interestingly, soluble molecules also induce changes in the cell count. Gunnarsson (1987) noted a reduction in the cell number of

locusts injected with β1,3-glucans derived from yeast cell walls. This stimulatory effect of β1,3-glucans is not restricted to insects since reduced cell counts have been found in other arthropods injected with glucans (Smith et al., 1984). Other soluble molecules which influence the cell count include bacterial lipopolysaccharide which elevates haemocyte counts in insects (Dunphy and Webster, 1988) and endotoxin from E. coli which reduces the cell count in Glossina (Kaaya et al., 1986).

The cellular events which underlie the gross changes in circulating cell number draw on information from virtually all aspects of cell behaviour. In the work presented here, the quantitative data obtained on cell number after stimulation provides an important insight to the immune response.

Changes in cell count may be simply quantitative, for example if cells marginated regardless of cell type. However, since populations of haemocytes are heterogeneous, the proportion of different types may also vary. Thus, the changes in cell count might also reflect qualitative alterations in the haemogram, for example if only one cell type dropped out of circulation, and this could have functional consequences to the immune response. The phagocytic competence of circulating cells may be considered to be an indicator or measure of qualitative change in the haemocyte populations. For example, Peake (1979) noted an increase in the phagocytic cell population in Calliphora with age. Several different approaches to determining the qualitative aspects of the immune response have been used in the present work and later chapters will report on these qualitative changes after immune manipulation.

An effective cellular response to parasitic invasion is often

characterised by an increase in circulating cell number and phagocytic uptake by haemocytes (Nappi, 1981), whereas successful parasitism is frequently accompanied by a decrease in haemocyte count (Guzo and Stoltz, 1987) and phagocytic uptake (Ibrahim et al., 1986). Therefore, the efficacy of the immune response can be considered to be a combination of the total cell count and the immunocompetence of the circulating cells. Information on both the number of phagocytic cells in circulation and the phagocytic capability of each provides clues to the competence of the immune system. Since phagocytosis is an effective mechanism for clearing the haemolymph of foreign particles, the phagocytic index is likely to indicate the extent of activation of the immune system in readiness for invasion.

An alteration in the haemocyte number may reflect changes in either one cell type eg. phagocytic cells, phenoloxidase-containing cells, or in all cell types. Several authors have found enhanced phagocytic uptake by haemocytes after immune activation (Mohrig et al., 1979a; Leonard et al., 1985a; Gunnarsson, 1987). An aim of this study was to use the in vivo phagocytic capacity as an indicator of immune responsiveness and identify whether the phagocytic cell type contributed to altered total cell counts. This work provided information on the proportions of phagocytic cells before and after stimulation and can be correlated with the results of previous studies on Hymenolepis infection in cockroaches (Holt, 1989a).

RESULTS

3.1. TOTAL HAEMOCYTE COUNTS

A variety of situations in which the THC varies were investigated using methods detailed in Section 2.2.1.

Zymosan A Supernatant (Zs) Injection

A saline injection induced a slight decrease in the circulating cell number compared with the THC in naive animals, but this change was only significant 12hr after treatment ($t=3.27$, $df=38$, $p<0.01$). Simply wounding the cuticle of the cockroaches could produce a change in the THC which persisted even 12hr after treatment ($t=3.5$, $df=38$, $p<0.01$).

Zs is stimulatory in the haemolymph of insects (Gunnarsson, 1988a). In these experiments, the THC was monitored at various time intervals after Zs injection to determine the dynamics of the immune response to the soluble molecules. The number of haemocytes circulating in the haemolymph of adult male P. americana after injection with Zs is shown in Fig. 3.1.1..

Intrahaemocoelic injection of Zs caused a rapid reduction in the cell number compared to controls, apparent after only 30min ($t=2.71$, $df=38$, $p<0.01$), which remained significantly lower ($t=3.74$, $df=41$, $p<0.01$) until 1hr after injection. At 3hr post-injection, although the THC was low, this difference was not significant. However, 12hr after injection of Zs there was a significant reduction in the number of circulating cells compared with naive animals ($t=5.3$, $df=34$, $p<0.01$).

3.1.2. Infection with H. diminuta

To find whether H. diminuta oncospheres were stimulating the immune

system during their attempts to penetrate the gut, the THC in experimental animals was monitored over several days after feeding. Animals mounting an immune response were expected to have a higher circulating haemocyte number.

Data from this experiment is shown in Fig. 3.1.2..

In cockroaches fed mature proglottids of H. diminuta, there was a significant ($t=2.87$, $df=34$, $p<0.01$) increase in the number of circulating blood cells after 1 day compared with controls. By 3 and 5 days after feeding, the THC had returned to a value not significantly different from the naive controls. Proglottids containing immature oncospheres were fed to cockroaches as controls and the THCs from these cockroaches were not significantly elevated when compared with the naive animals.

The results suggest that live oncospheres of Hymenolepis stimulate an increase in the number of cells available to participate in the immune response.

3.1.3. Infection with M. moniliformis

Acanthors of M. moniliformis damage the gut during their passage to the haemocoel. It was expected that the cockroaches infected orally with the parasite would be stimulated to mount an immune response to the parasite and the damaged gut tissue. Hosts with activated immune systems would be expected to have recruited cells into the haemolymph in preparation for infection.

The results are presented in Fig. 3.1.3.a.

The THCs were reduced in cockroaches fed either live ($t=2.7$, $df=36$, $p<0.05$) or heat-inactivated ($t=2.83$, $df=28$, $p<0.01$) acanthors of

Moniliformis 1 day previously compared with naive animals. By 3 days after feeding, the haemocyte count had returned to resting levels in the cockroaches fed with live ($t=0.01$, $df=32$, $p>0.05$) and dead ($t=1.35$, $df=27$, $p>0.05$) acanthors compared with naive cockroaches, suggesting that the stimulatory effect of the parasite on the immune system was short-lived. The drop in THC after feeding with dead acanthors could be caused by a few acanthors which had survived the heat treatment burrowing through the gut.

Since cockroaches were responding to the initial stages of infection with M.moniliformis by decreasing the circulating haemocyte number, the THCs from cockroaches with established Moniliformis infections of known dose were investigated to find whether a low cell count was permitting parasite survival.

Haemocyte counts from adult, female cockroaches experimentally infected with a known dose of M. moniliformis acanthellae were unaltered suggesting that this parasite does not survive in the haemocoel by depressing the host circulating cell number (see Fig. 3.1.3.b). Control animals received injections of medium only or medium containing ovariole. The latter remains unencapsulated in allografts and was used in these experiments to mimic the presence of the acanthellae within the haemocoel. Neither of the control treatments altered the cell number compared with naive female cockroaches.

To find whether there was a relationship between the number of circulating cells and parasite survival, the cockroaches used in the THC experiment above were dissected after the assay and the parasites recovered. Figs. 3.1.3.c-d, scatter graphs, show the total cell count versus the number of viable parasites. There was no correlation in

either experiment between the THC and the number of parasites recovered using Spearman's rank correlation test (Exp. 1 - n=19, Spearman's r=0.163, p>0.05; Exp. 2 - n=19, Spearman's r=0.004, p>0.99). This shows that the survival of experimentally injected Moniliformis acanthellae is entirely independent of the circulating cell number.

3.1.4. Abiotic Particles

Fig. 3.1.4. represents data collected from cockroaches injected with FITC-labelled latex spheres, abiotic particulate material which is readily phagocytosed by haemocytes (see Fig. 3.2.2.). Latex spheres caused a rapid and substantial decrease in the circulating cell number only 30min after injection ($F=11.33$, $df=2,57$, $p<0.01$). One hour after treatment, the circulating cell population was still significantly depleted ($F=5.92$, $df=2,58$, $p<0.01$) but 2hr after injection, the THC was not significantly lower than controls.

3.2. PHAGOCYTOSIS

3.2.1. Identification of the Haemocyte Types in P. americana

Haemocytes fixed in vivo or in suspension after haemolymph collection cannot readily be identified as belonging to either the plasmatocyte or coagulocyte cell type because of the similarities in their morphology. To identify the main haemocyte types by phase contrast microscopy, cells must be allowed to adhere to and spread on the substratum. Haemocyte types are shown on Plates 3.2.1.a-b.

Using Price and Ratcliffe's (1974) criteria, plasmatocytes can be distinguished as those cells which spread extensively on many surfaces. The coagulocyte does not spread and in vitro this cell type lyses and

degranulates, and is often identifiable as a nucleus surrounded by remnants of cytoplasm and attached to the slide by a diffuse material. In the fresh state, these cells appear phase bright, due to vacuoles around the nucleus and are easily distinguished from the large, spread and phase dark plasmatocytes. In Periplaneta, plasmatocytes often contain variable numbers of granules usually with a perinuclear distribution.

3.2.2. Time Course of Phagocytic Uptake of Abiotic Particles In Vivo

Figure 3.2.2. and Plates 3.2.1.a-b represent data collected in this experiment.

The in vivo phagocytic uptake of FITC labelled latex spheres by haemocytes from naive cockroaches indicates that the plasmatocytes are the main phagocytic cell type, although latex spheres were found associated with coagulocytes. Latex spheres phagocytosed by plasmatocytes were usually perinuclear in location and apparently accumulated into several vacuoles which ranged in size (see Plate 3.2.2). The degree of phagocytic uptake was not consistent within the plasmatocyte cell type. Many plasmatocytes did not contain any latex spheres while others may have internalised only a few beads or up to several hundred. Similar population heterogeneity has been reported in mammalian phagocytes (Sheterline and Rickard, 1984).

A preliminary experiment was carried out to define the time at which phagocytic uptake is highest (see Fig. 3.2.2). Over 10% of plasmatocytes had phagocytosed beads 2hr ($t=3.96$, $df=17$, $p<0.01$ compared with time 0 using Student's t-test) after injection of the latex spheres and this remained quite consistent up to 6hr after injection, after which the values tailed off gradually.

Although phagocytosis occurred remarkably rapidly, some cells having acquired beads before the first collection (time 0), it is likely that the haemocytes endocytosed these beads during the time required for collection and spreading of the cells. In subsequent experiments on phagocytosis, a 2hr time interval after injection was allowed before collection of haemolymph samples.

3.2.3. Zymosan A Supernatant Injection

A 3hr Zs pretreatment did not stimulate phagocytic uptake of fluorescently labelled latex spheres (see Fig. 3.2.3.a). There was no change in the total percentage of phagocytic cells compared with naive or saline-injected control animals ($F=1.63$, $df=2,61$, $p>0.05$). Similarly, the number of beads phagocytosed by each cell did not change after Zs treatment.

A Zs injection 12hr before the phagocytosis assay had no effect on either the total number of phagocytic cells or the proportions of the weakly or strongly phagocytic subpopulations (see Fig. 3.2.3.b).

3.2.4. Infection with *H. diminuta*

Fig. 3.2.4.a-c represents data collected on phagocytosis of latex spheres by plasmatocytes from animals fed with *H. diminuta* several days previously.

In naive animals around 6% of plasmatocytes were phagocytic, of which approximately 5% contained few beads. After feeding with *Hymenolepis*, the percentage of plasmatocytes which were phagocytic increased. On day 1 after feeding the number of phagocytic plasmatocytes had more than doubled (Exps. 2 and 3) compared with cells from the naive animals, and

quadrupled in Exp. 1. Most of the phagocytic plasmatocytes had taken up few beads (less than 25 beads).However, there was an increase in the percentage of very phagocytic cells (more than 100 beads). In each replicate, there was a small increase in the percentage of phagocytic haemocytes from animals fed immature Hymenolepis compared to the naive animals. This was not a significant change and is possibly due either to handling of the animals during feeding or to the presence of a small number of mature oncospheres in the proglottids fed to the controls.

Analysis of the pooled results indicated that there was a highly significant increase in the number of phagocytic cells after feeding with the parasite ($F=28.83$, $df=2,59$, $p<0.01$). This could be attributed to a significant increase in both the highly phagocytic cell population ($F=21.95$, $df=2,59$, $p<0.01$) and the less phagocytic population ($F=25.55$, $df=2,59$, $p<0.01$). This pattern occurred in all replicates, although most notably in Exp. 1. This may be due to slight differences in experimental conditions but is more likely to be caused by variable crop emptying rates altering the movement of the oncospheres along the gut. If the crop emptying rate is too slow then the parasite may die through lack of nutrients before reaching the midgut so that there will be no immune stimulation by hatching oncospheres.

On day 2 after feeding the response to the Hymenolepis was lower than previously. Haemocytes from cockroaches fed mature proglottids had twice as many phagocytic plasmatocytes in their haemolymph compared with naive animals. Analysis of variance (ANOVA) revealed that the increase in phagocytic cells was significant ($F=15.12$, $df=2,60$, $p<0.01$) and was caused by a significant increase in the number of cells with few beads ($F=14.7$, $df=2,60$, $p<0.05$) and a small non-significant

increase in the number of highly phagocytic cells.

Three days after feeding, more weakly phagocytic cells remained in circulation ($F=4.19$, $df=2,61$, $p<0.05$) compared with controls. However, the total number of phagocytic cells in the animals fed mature Hymenolepis had declined to approximately control levels. After 5 days, the phagocytic capacity of the immune system was equivalent to that of the controls. There was no change in the total number of phagocytic cells ($F=1.53$, $df=2,18$, $p>0.05$) or in the subpopulations of phagocytic cells.

In summary, mature oncospheres increase the phagocytic capacity of the immune system. The stimulatory effect is highest 1 day after feeding and declines gradually, until 5 days after feeding when phagocytic uptake has returned to control values.

3.3. NODULE FORMATION

3.3.1. Zymosan A supernatant Injection

The results are given in Figs. 3.3.1.a-b.

Cell aggregation was measured 3hr after injection of Zs into the haemocoel of cockroaches. The number of nodules was significantly different after Zs injection from all other treatments in each of the repeated experiments ($t=6.18$, $df=20$, $p<0.01$ for pooled data from 3 experiments), indicating that Zs stimulates the cells of the immune system.

The haemolymph of naive animals contained few nodules (see Fig. 3.3.1.) and wounding the cuticle and epidermis of the insect or injecting saline into the haemocoel did not alter the number of nodules in the haemolymph.

After 12hr few nodules remained in circulation (see Fig. 3.3.1.b). A single saline injection did not stimulate a significant increase in the number of nodules formed. However, animals treated with Zs 12hr previously, then injected with saline, had a significantly larger number of nodules ($t=7.62$, $df=19$, $p<0.001$ for pooled data) than expected compared with those receiving only a saline injection. This is a non-specific stimulation, possibly due to wounding, of an already activated and responsive immune system.

3.3.2. Infection with Acanthors of M. moniliformis

Fig. 3.3.2. represents data from an experiment on nodule formation designed to determine whether Moniliformis stimulates the immune system of its cockroach host as it burrows through the gut and emerges in the haemocoel.

Shelled acanthors of M. moniliformis were fed to adult male cockroaches and the number of nodules in the haemolymph was assayed two days later. Acanthors were frequently found in haemolymph samples, indicating that hatching and gut penetration had occurred. These acanthors were occasionally encapsulated and a few were melanised. Cockroaches fed heat-inactivated acanthors had a similar number of nodules in their haemolymph compared to the naive animals. However, animals fed live M. moniliformis acanthors had a larger, but not significantly different, number of cell aggregates present in their haemolymph. This suggests that even in the initial stages of gut penetration and establishment within the cockroach haemocoel, the parasite has a limited effect on the immune system of the host.

Plate 3.2.1.a

Phase contrast micrograph of haemocytes from P. americana after 30min in vitro on glass. Scale bar = 100µm

PL = plasmatocyte, uPL = unspread plasmatocyte
CO = coagulocyte
F = filopodium
L = lamellipodium
arrows = granules, arrowheads = vacuoles

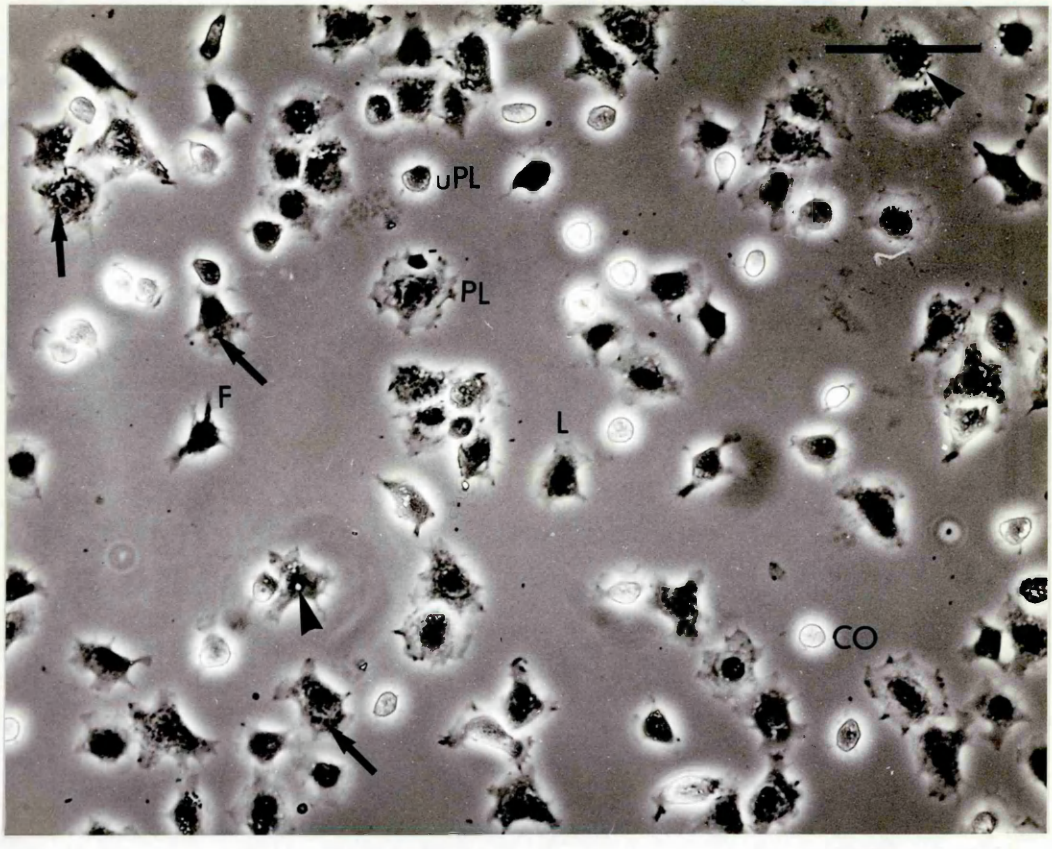


Plate 3.2.1.b

Fluorescence micrograph of haemocytes from P. americana which have phagocytosed FITC-latex spheres in vivo. Phagocytic cells spread after 30min in vitro on glass and are plasmatocytes. Scale bar = 30μm

PL = plasmatocyte
arrows = phagocytosed latex particles

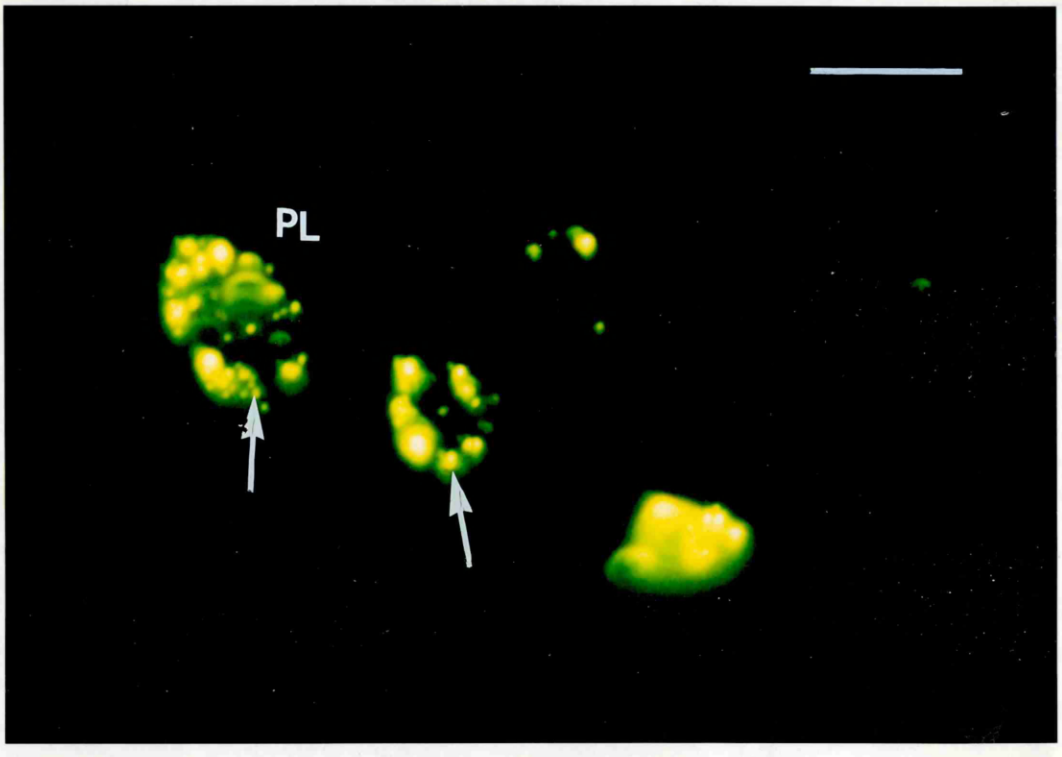


FIG. 3.1.1.a - HAEMOCYTE COUNTS AFTER ZYMOSAN SUPERNATANT INJECTION

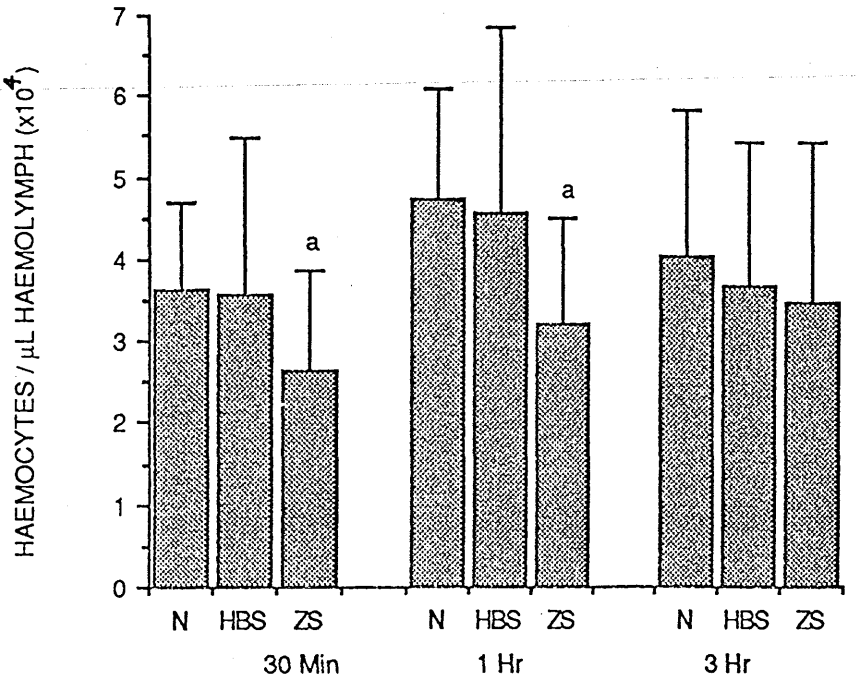


Figure 3.1.1.a
Changes in total haemocyte count in adult male Periplaneta americana at various time intervals after injection of Zymosan A supernatant. Results are expressed as the mean number of haemocytes / µl haemolymph x10 ^{4} + s.d.. Data from 18-20 animals / treatment. N = naive, HBS = saline, Zs = Zymosan A supernatant.
a = $p < 0.01$ using twosample t-test, compared to naive control.

Fig. 3.1.1.b - HAEMOCYTE COUNTS 12HR AFTER ZYMOSAN SUPERNATANT INJECTION

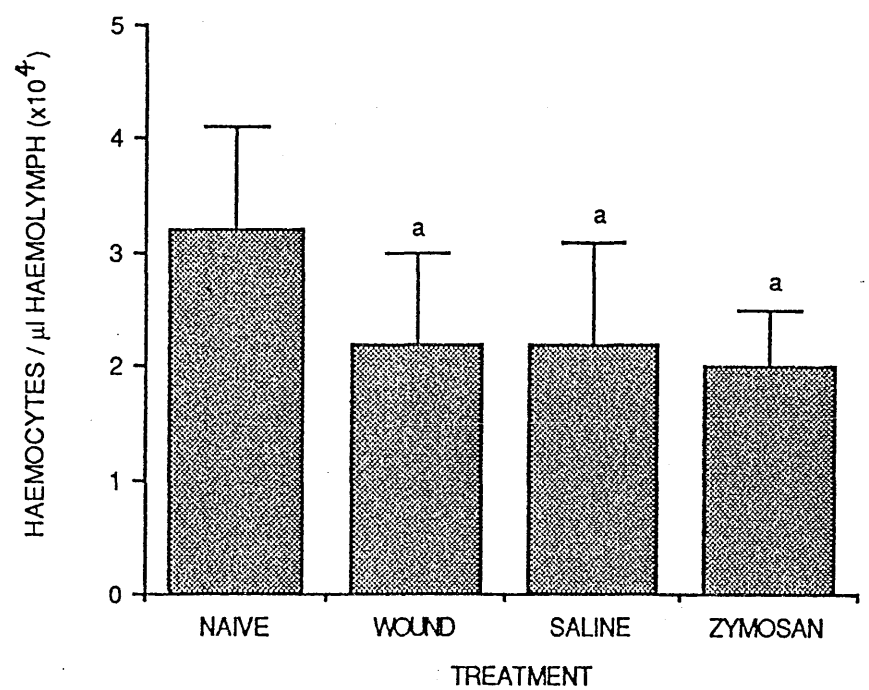


Figure 3.1.1.b.
Changes in total haemocyte count 12 hr after injection of Zymosan A supernatant . Results are expressed as the mean + s.d haemocytes / µl haemolymph x10 ^{4} . Data from 20 animals / treatment.
a = $p < 0.01$ using twosample t-test, compared to naive control.

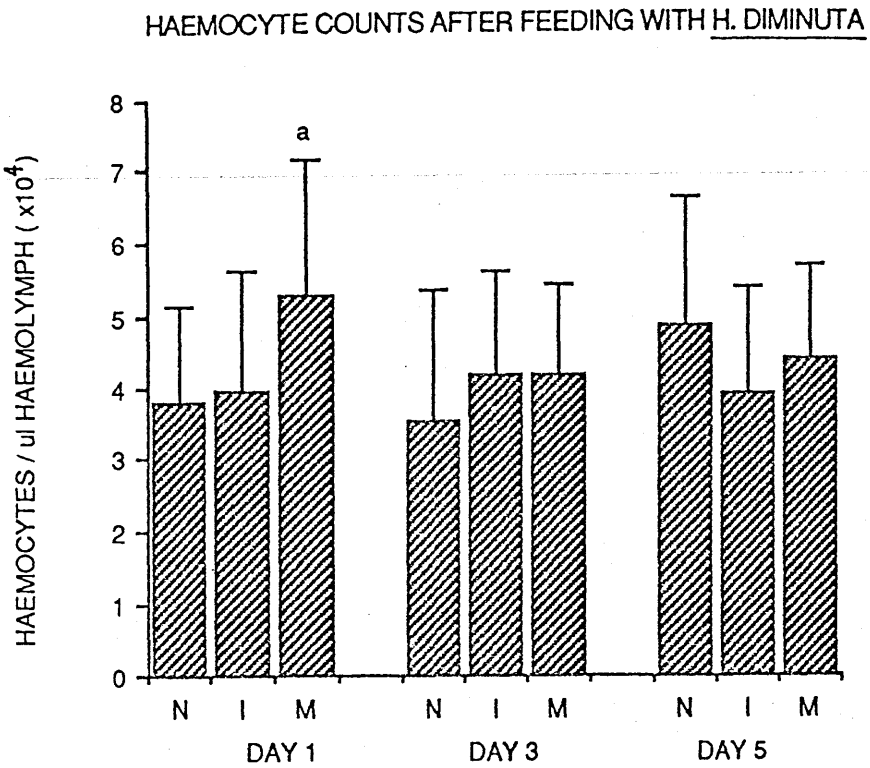


Figure 3.1.2.
Changes in total haemocyte count in adult male Periplaneta americana 1,3 and 5 days after feeding with proglottids of Hymenolepis diminuta. Results are expressed as the mean number of haemocytes / µl haemolymph x10 ^{4} ± s.d.. Data from 18-20 animals / treatment. N = naive, I = immature Hymenolepis, M = mature Hymenolepis.
a = $p < 0.01$ using twosample t-test, compared to naive control.

FIG. 3.1.3.a - HAEMOCYTE COUNTS AFTER FEEDING WITH M.MONILIFORMIS

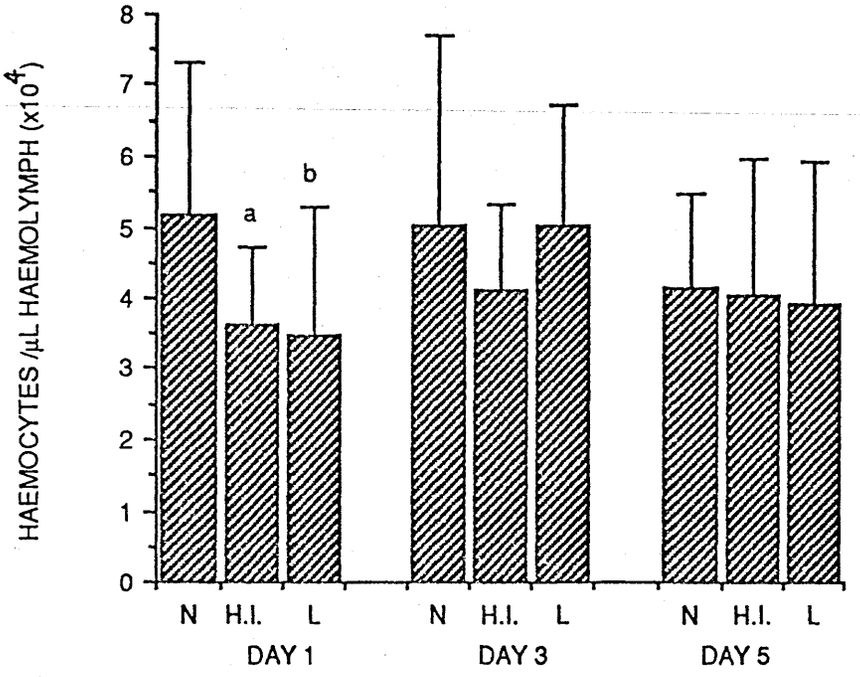


Figure 3.1.3.a
Changes in total haemocyte count in adult male Periplaneta americana 1,3 and 5 days after feeding with shelled acanthors of Moniliformis moniliformis. Results are expressed as the mean number of haemocytes / µl haemolymph x10 ^{4} ± s.d.. Data from 20 animals / treatment.
N = naive control, H.I. = heat-inactivated Moniliformis, L = live Moniliformis.
a = $p < 0.01$ using twosample t-test, compared to naive control.
b= $p < 0.05$ using twosample t-test, compared to naive control.

FIG. 3.1.3.b - HAEMOCYTE COUNTS AFTER INFECTION WITH M.MONILIFORMIS ACANTHELLAE

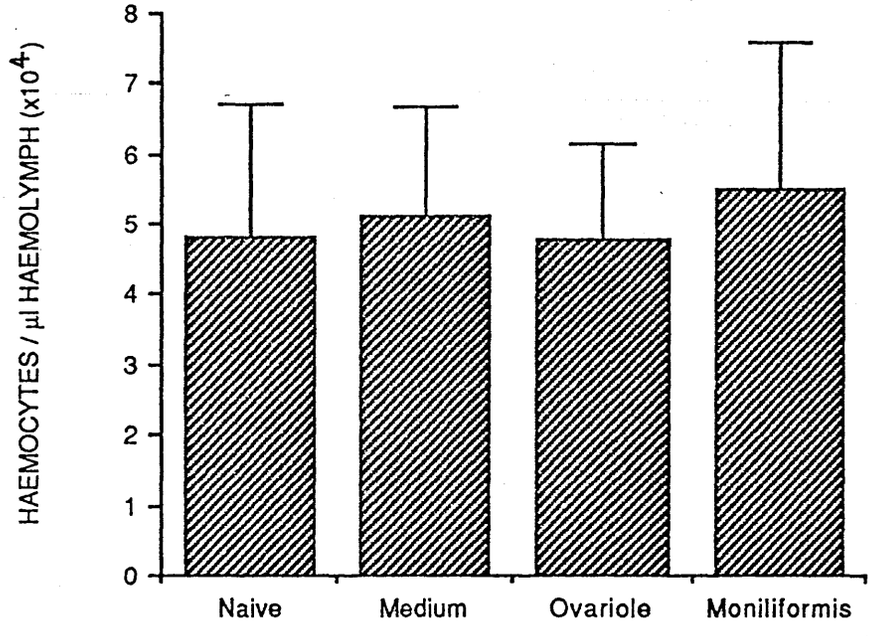


Figure 3.1.3.b
Changes in the total haemocyte count after infection with 40 acanthellae II of Moniliformis moniliformis. Results are expressed as the mean ± s.d. haemocytes / µl haemolymph x10 ^{4} . Data from 20 animals / treatment.
All values non-significant compared to naive control using twosample t-test.

FIG. 3.1.3.c (EXP 1) = RELATIONSHIP BETWEEN PARASITE NUMBER AND CELL COUNT

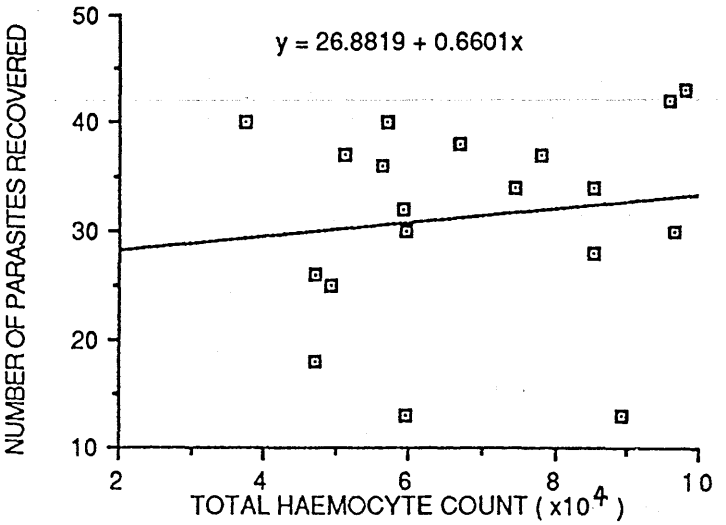
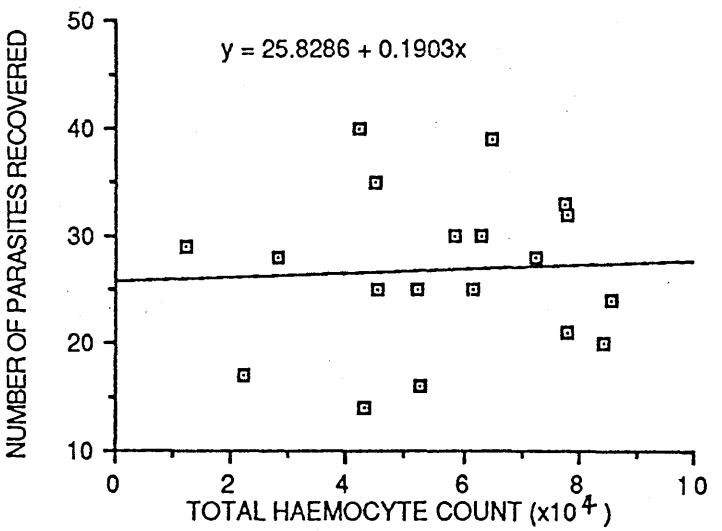


FIGURE 3.1.3.d (EXP 2)



Figures 3.1.3.c and d
Scatter diagrams to represent the relationship between the total haemocyte count and the survival of Moniliformis moniliformis acanthellae II in experimentally infected cockroaches. Haemocyte counts are expressed as the mean number of haemocytes / µl haemolymph $x10^4$. The equation for the line of best fit is given for each experiment.

Table 3.1.4 - CELL COUNTS AFTER INJECTION WITH FITC-LATEX

Total haemocyte count (THC) (number of haemocytes/µl haemolymph; mean+s.d.) in adult female P. americana injected with FITC-latex (10µl 2% in HBS) or HBS (10µl). Number of animals in brackets. ND=not done
a=p<0.01 using Student's t-test

TIME P.I. (hr)	THC/µl HAEMOLYMPH ($x10^4$) Naive	Saline	FITC-latex
0	4.68+1.19 (20)	4.5+0.9 (20)	4.8+2.2 (18)
15	ND	5.0+1.7 (20)	ND
30min	4.39+1.28 (20)	3.7+1.4 (20)	2.4+1.1 (20)a
1	4.31+1.45 (20)	4.0+1.2 (20)	2.9+1.4 (20)a
2	4.22+1.24 (20)	4.4+2.2 (20)	3.8+1.7 (20)
3	ND	3.7+1.6 (20)	3.0+0.9 (18)a
4	3.82+1.14 (20)	4.1+1.4 (20)	3.5+1.5 (10)
5	ND	4.1+1.2 (20)	3.6+1.9 (9)
6	3.91+1.48 (20)	3.5+1.3 (20)	2.9+0.8 (11)
7	ND	ND	2.6+1.1 (10)

FIG. 3.1.4.a - HAEMOCYTE COUNTS AFTER INJECTION OF LATEX SPHERES

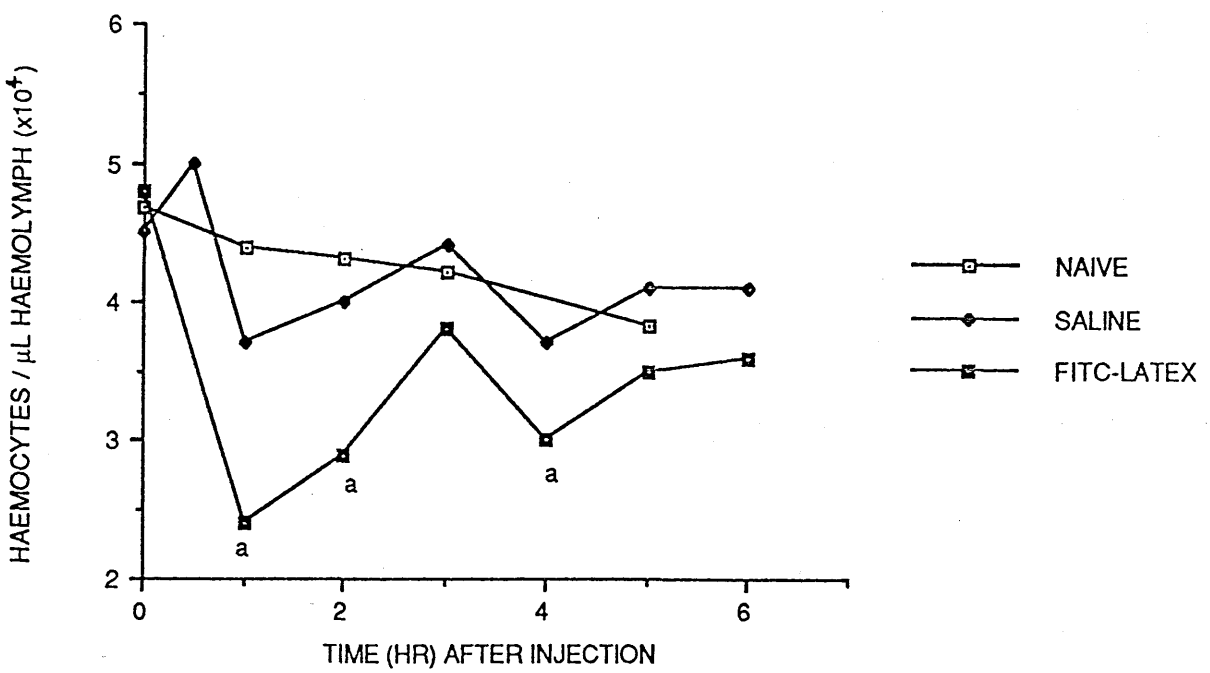


Figure 3.1.4.a
Changes in total haemocyte count in Periplaneta americana vs time after injection of saline or FITC-latex spheres. Results are expressed as the mean number of haemocytes / µl haemolymph x10 ^{4} ± s.d.. Data from 20 animals / treatment.
a = p < 0.01 using twosample t-test, compared to naive control.

FIG. 3.2.2. - PHAGOCYTIC UPTAKE BY HAEMOCYTES OF P.AMERICANA

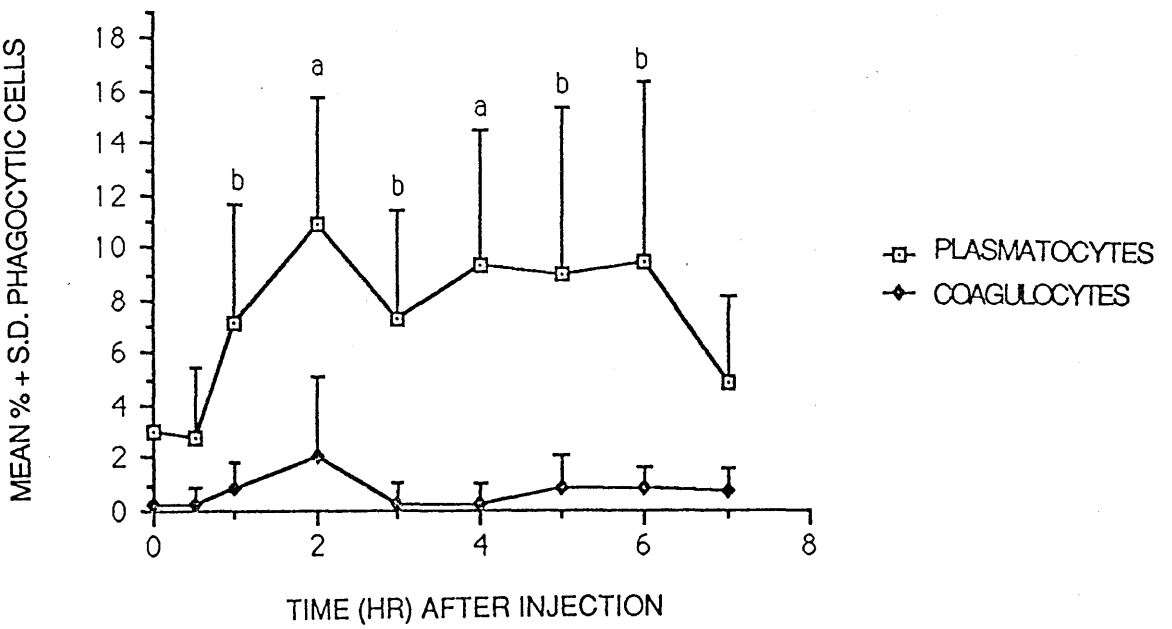


Figure 3.2.2.
In vivo phagocytic uptake of FITC- latex spheres by haemocytes of adult Periplaneta americana vs time after injection with beads. Results are expressed as the mean % ± s.d cells associated with beads, based on examination of 200-300 cells/ insect. Data from > 8 animals / time interval.
a=$p<0.01$ compared with time 0 using Student's twosample t-test.
b=$p<0.05$ compared with time 0 using Student's twosample t-test.

FIG. 3.2.3.a - PHAGOCYTOSIS 3HR AFTER ZYMOSAN PRE-TREATMENT

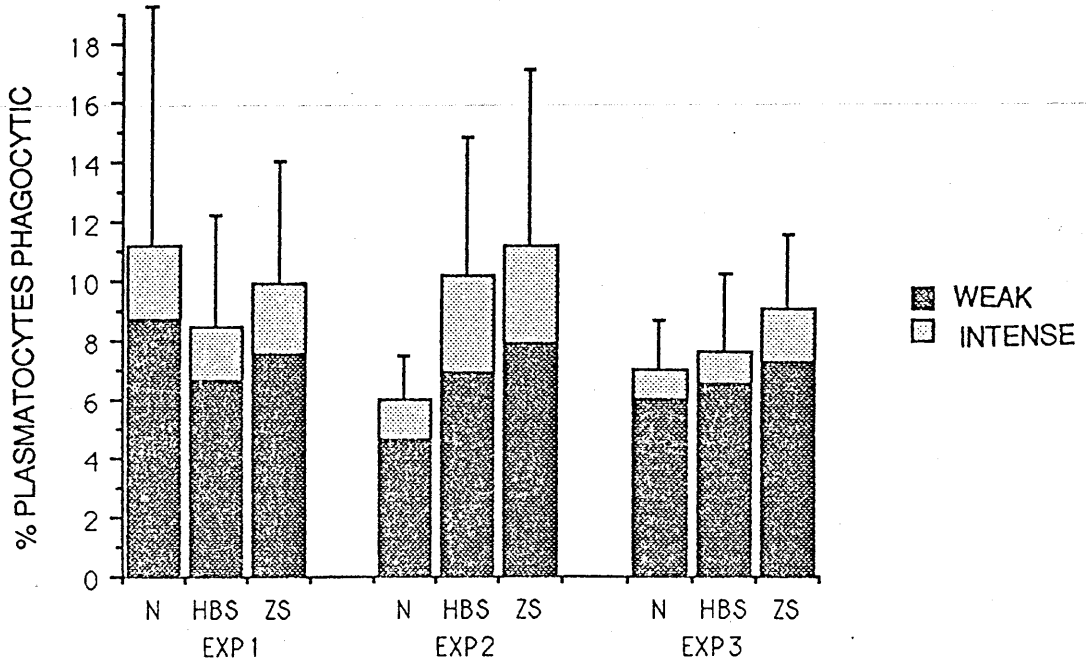
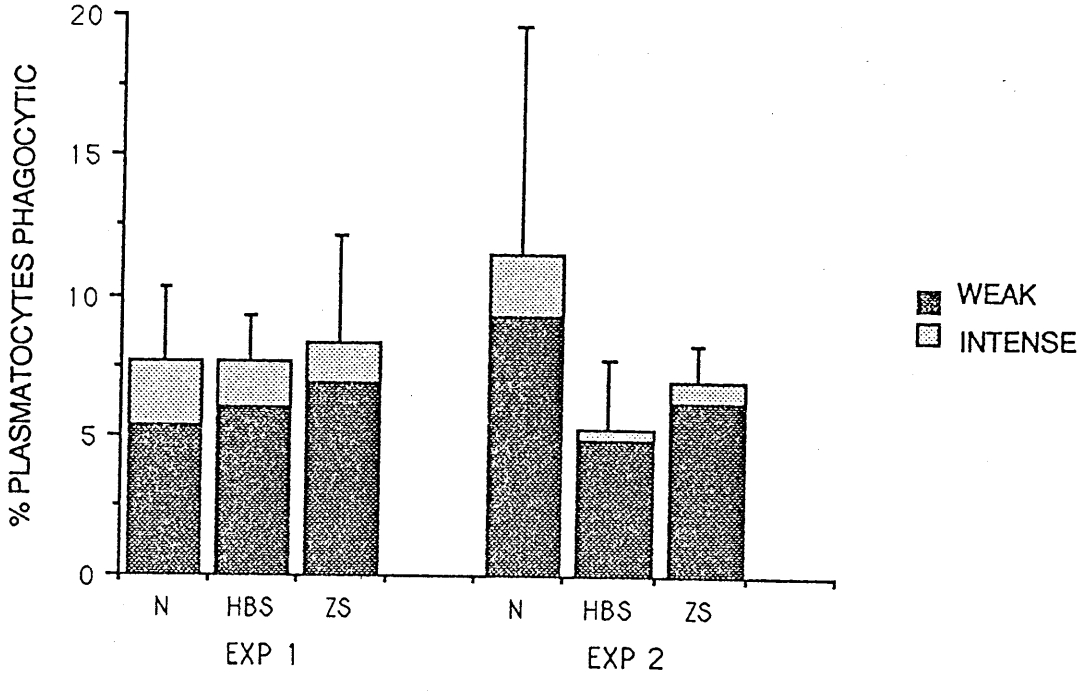
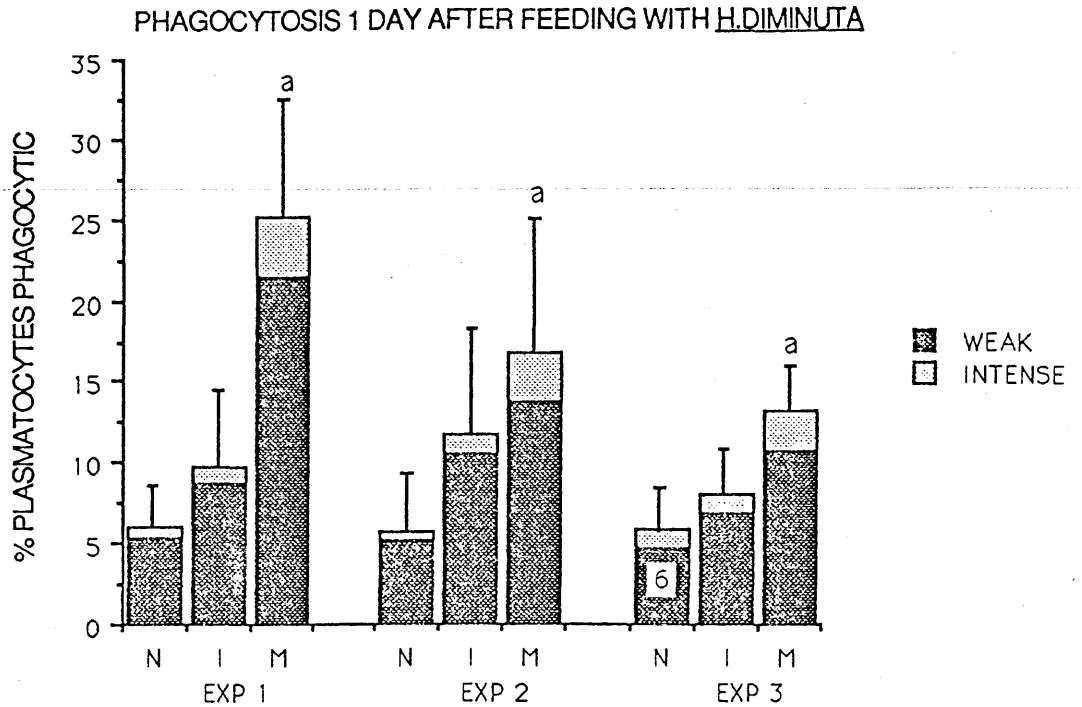


FIG. 3.2.3.b - PHAGOCYTOSIS 12HR AFTER ZYMOSAN PRE-TREATMENT



Figures 3.2.3.a and b
In vivo phagocytic uptake of FITC-latex spheres by haemocytes of adult Periplaneta americana 3 and 12 hours after injection of Zymosan A supernatant (10µl 2% in HBS). Results are expressed as the mean % ± s.d plasmatocytes associated with beads, based on examination of 200-300 cells/ animal. Data from 6-7 animals/ treatment.
N = naive, HBS=saline injection, Zs=Zymosan A supernatant.
All values non-significant, using Student's twosample t-test.



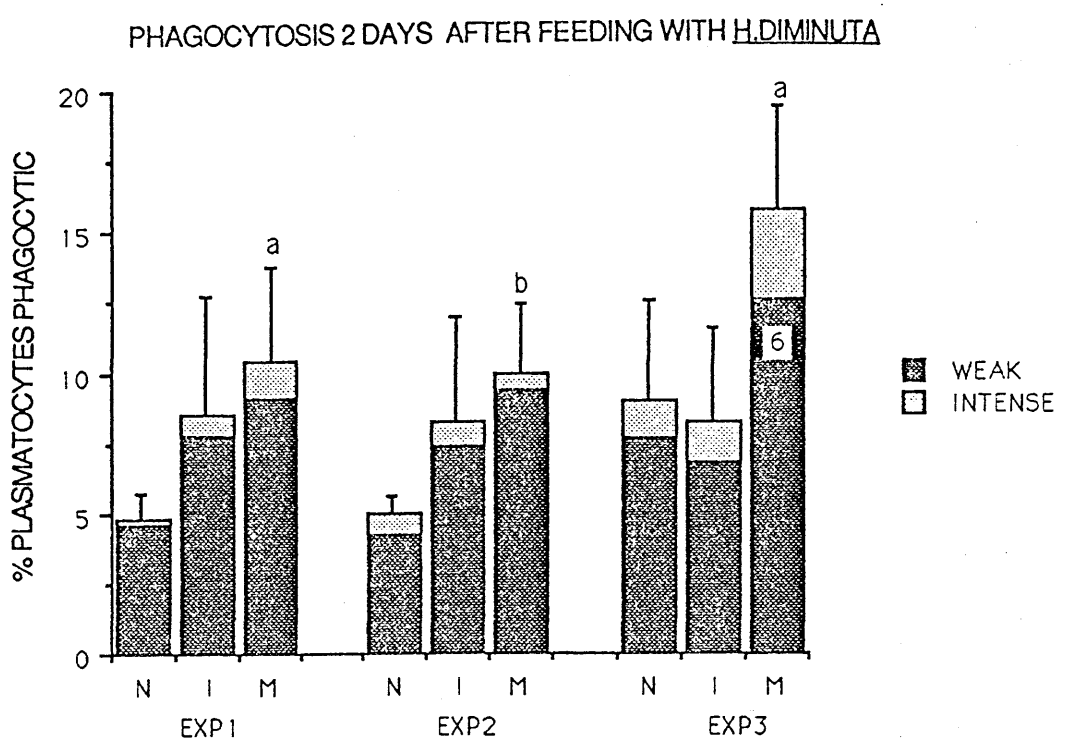


Figure 3.2.4.a and b

In vivo phagocytic uptake of FITC-latex spheres by haemocytes 1and 2 days after feeding with Hymenolepis diminuta. Results are expressed as the mean % ± s.d plasmatocytes associated with beads, based on examination of 200-300 cells/ animal. Data from 7 animals/ treatment, except where stated.

N = naive, I = immature Hymenolepis, M = mature Hymenolepis.

a = $p < 0.01$ using twosample t-test, compared to naive control.

b= $p < 0.05$ using twosample t-test, compared to naive control.

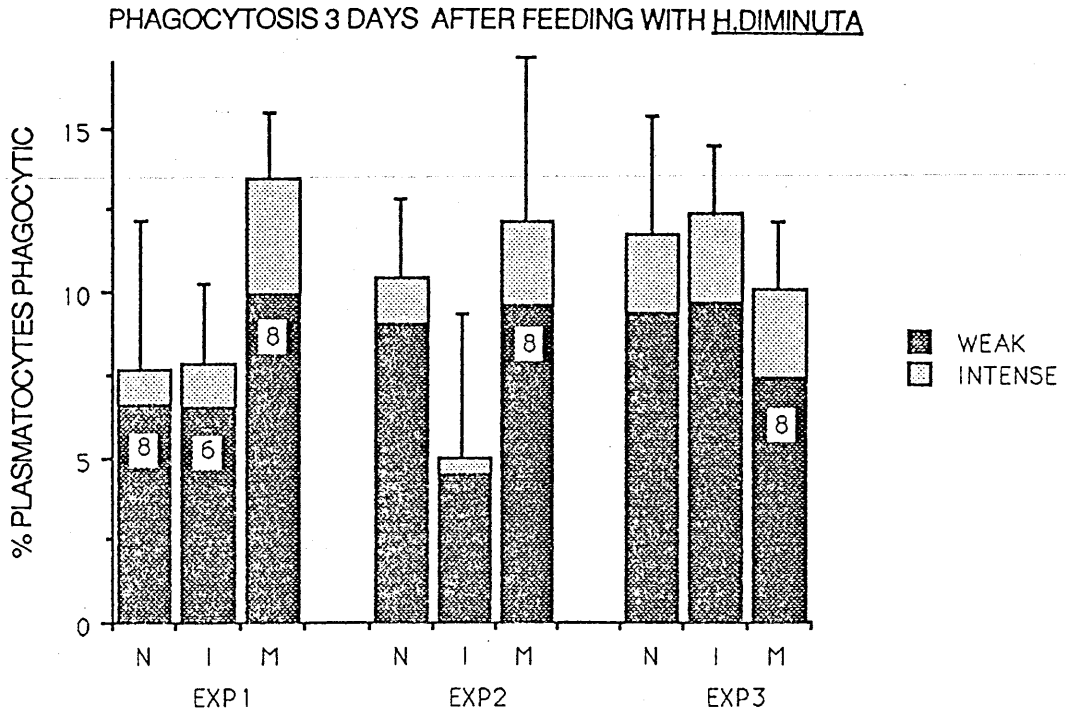


Figure 3.2.4.c

In vivo phagocytic uptake of FITC- latex spheres by haemocytes 3 days after feeding with Hymenolepis diminuta. Results are expressed as the mean % ± s.d plasmatocytes associated with beads, based on examination of 200-300 cells/ insect. Data from 7 animals/ treatment, except where stated.

N = naive, I = immature Hymenolepis, M = mature Hymenolepis.

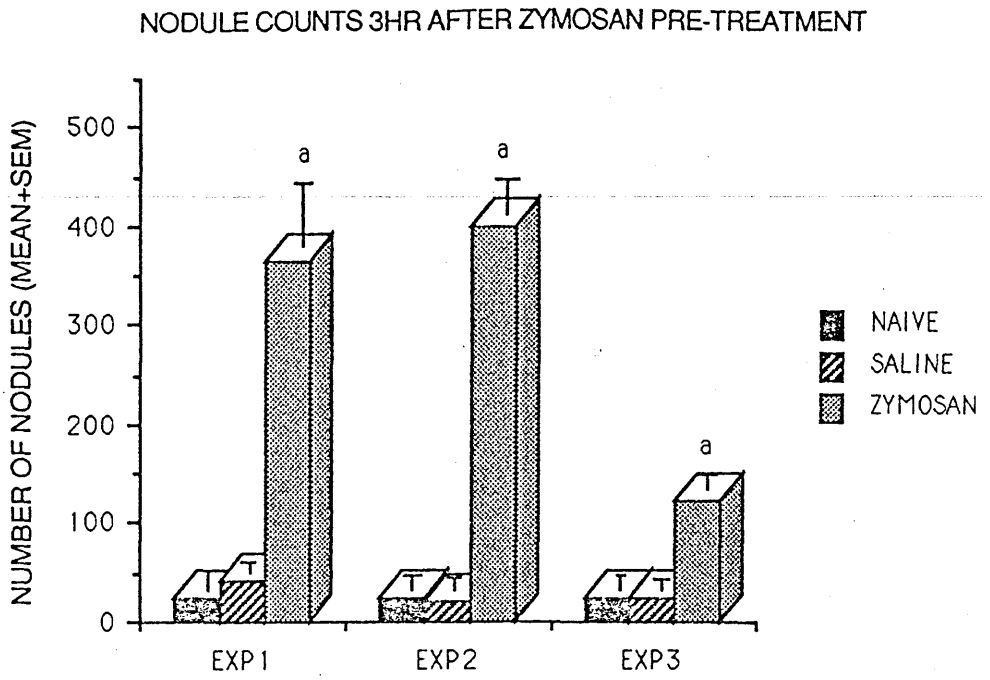


Figure 3.3.1.a
Nodule formation in adult male Periplaneta americana 3 hr after injection of Zymosan A supernatant. Results are expressed as the mean ± s.e.m. number of nodules. Data from 7 animals / treatment, except where stated.
a = $p < 0.01$ using twosample t-test, compared to naive control.

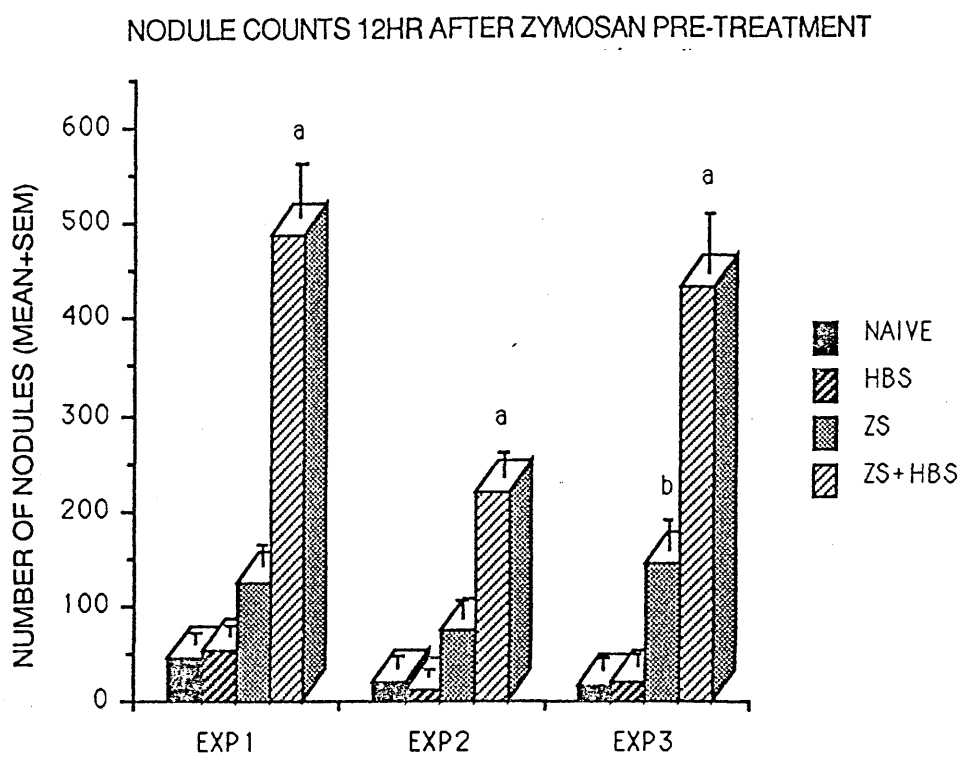


Figure 3.3.1.b
Nodule formation in adult male Periplaneta americana 12hr after injection of Zymosan A supernatant. One group received an injection of saline 12hr after Zymosan treatment and nodules were collected 3hr later. Results are expressed as the mean ± s.e.m. number of nodules. Data from 7 animals / treatment.
HBS = saline control, Zs = Zymosan , Zs+HBS = Zymosan (12hr) + saline (3hr)
a = $p < 0.01$ using twosample t-test, compared to naive control.
b = $p < 0.02$ using twosample t-test, compared to naive control.

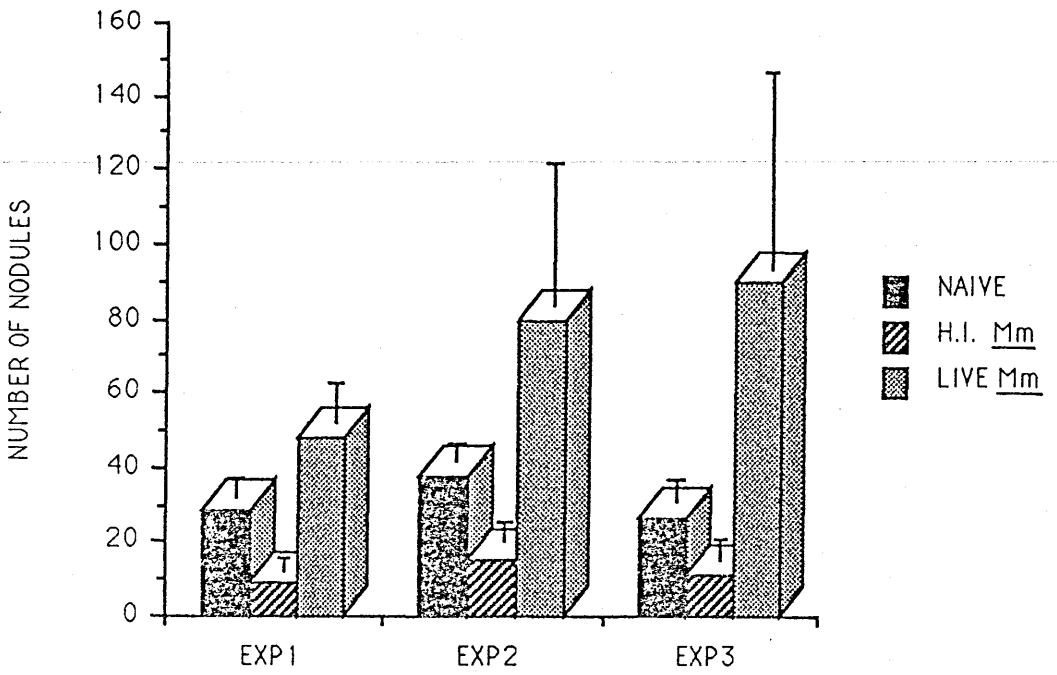


Figure 3.3.2.
Nodule formation in adult male Periplaneta americana fed with shelled acanthors of Moniliformis moniliformis 2 days previously. Results are expressed as the mean ± s.e.m. number of nodules. Data from > 5 animals / treatment.
N = naive control, H.I. Mm = heat-inactivated Moniliformis, Live Mm = viable Moniliformis.
All values non-significant using twosample t-test compared to naive control.

DISCUSSION

Several assays have been used to assess the immunocompetence of insects after exposure to various stimuli, either stimulatory or inhibitory. The circulating cell number, the in vivo phagocytic capacity of the cells and the ability to form nodules (Ratcliffe and Walters, 1983; Gunnarsson and Lackie, 1985) have all been studied.

The THC in P. americana varies considerably between naive individuals of the same sex (Fig. 3.1.1.a) and between sexes (Lackie et al., 1985) and the literature reflects these differences. Gupta and Sutherland (1966) report a very low cell count of between $0.8\text{-}2.7\text{x}10^4$ cells/μl^{-1} haemolymph whereas Tauber and Yeager (1935) quote much higher values ($5.1\text{x}10^4$ cells/μl^{-1}) which are comparable with those of Ryan and Nicholas (1972). Takle (1986) found a cell count of around $5.0\text{x}10^4$ cells/μl^{-1} for male adults of P. americana. The results in the present work agree quite closely to the latter, haemocyte counts from adult male cockroaches varying between $3.5\text{-}5.5\text{x}10^4$ cells/μl^{-1} haemolymph.

The THC of insects is a dynamic parameter which is readily perturbed by damage to the cuticle. Wounding the cuticle of the insect by saline injection caused a small and transient change in the circulating cell number (Fig. 3.1.1.b). Dunphy et al. (1986) reported a short-lived decrease in the number of circulating haemocytes in experimentally wounded or saline-injected Galleria mellonella and Gunnarsson (1988a) observed a similar effect in locusts. Wound factors released from the damaged site alter cell morphology and adhesiveness (Cherbas, 1973) and may mediate the initial responses to invasion, including the cell number.

The insect THC has been found to be altered by bacterial (Dunphy et

al., 1986) and fungal infection (Dunphy and Nolan, 1982) in addition to soluble molecules derived from microbes (Gunnarsson, 1988a). In the work presented here, Zs supernatant containing the stimulatory carbohydrates β1,3-glucans from yeast cell walls (Bacon et al., 1969) rapidly reduced the cell number but the effect was short-lived, returning to resting levels after only 3hr (Fig. 3.1.1). Gunnarsson (1988a) noted a similar decline in the THC of Schistocerca gregaria in response to Zs. Since nodule formation in insects is also induced by Zs treatment (Gunnarsson and Lackie, 1985; Figs. 3.3.1.a-b) it is likely that the decline in THC occurs as haemocytes form nodules. However, Zs injection increases the immunocompetence of cockroach haemocytes since there is an enhanced nodule response to a non-specific stimulant even after several hours (Fig. 3.3.1.b).

In contrast with Zs, the circulating blood cell number of cockroaches increases on infection with Hymenolepis (Fig. 3.1.2). After a period of days, the THC returns to normal as the oncospheres pass along the gut and the stimulus is removed. Other examples exist in the literature of elevated blood cell numbers in insects which are undergoing successful defence reactions. Nappi and Stoffolano (1971) noted a high THC in the dipteran fly, Musca domestica during the immune response to Heterotylenchus autumnalis and Nappi (1981) found more blood cells in Drosophila melanogaster infected with eggs of the braconid wasp, Asobara tabida. In a recent study, Christensen et al. (1989) reported significantly elevated cell counts in mosquitoes encapsulating microfilariae of Dirofilariae immitus.

However, any haemocytic response of the host to the invader should be recognised as a complex interaction between the host defences and the ability of the parasite to evade or suppress those defences. Thus, a

parasite may stimulate changes in the host haemogram yet still be able to survive.

Acanthors of Moniliformis penetrate the host intestine very rapidly and are found on the haemocoelic side only one day after exposure (Rotheram and Crompton, 1972). Infected cockroaches have fewer circulating cells (Fig. 3.1.3.a) and more haemocytic aggregates (Fig. 3.3.2) suggesting that the host immune system responds to the initial stages of infection with the parasite larvae even though later developmental stages do not seem to be stimulatory. Interestingly, the THC quickly returned to resting levels even though the stimulus ie. the acanthors, remained in the haemocoel. Robinson and Strickland (1969) found that haemocytic encapsulation of acanthors was unsuccessful and haemocytes dropped away after several days. These results could indicate the onset of immune evasion by the parasite as the haemocytic stimulus is perhaps removed or masked.

It has been suggested (Lackie and Holt, 1988) that Moniliformis immunosuppresses the cockroach host to avoid cellular attack during the parasite development in the haemocoel. Perhaps the simplest mechanism for parasite survival would be to reduce the number of host effector cells. However, the THC was unaffected in acanthellae-infected cockroaches which supports the hypothesis of non-recognition, or immune evasion, rather than immune suppression. On the otherhand, the parasite could avoid the immune response by inhibiting cell behaviour rather than cell number. Finally, the parasite could actively evade recognition by a competent immune system with a full complement of effector cells. The present results support the latter hypothesis although Lackie and Holt (1988) provided a strong case for immune

suppression. These authors found a reduced capacity for β1,3-glucan-induced phagocytosis and nodule formation in haemocytes from Moniliformis-infected cockroaches. The results of cell behavioural assays which test host immunocompetence will be reported in later chapters to try to clarify the question of immune evasion versus suppression.

The number of surviving acanthellae was unaffected by the host cell count. It appears then that it is the gut not the immune system which is responsible for the huge parasite losses and the subsequent variability in the intensities of infection found in orally infected cockroaches (Lackie, 1972b). Parameters such as crop emptying rates might influence parasite infectivity by exhausting the parasites of nutrients before penetration can occur. These results are relevant to studies of parasite dispersion in the host population since it is often difficult to distinguish between the importance of the gut barrier compared with the immune response relative to host infection rates.

Changes in the THC may arise from changes in one population or in all types of cells. Gagen and Ratcliffe (1976) reported a rapid and dramatic decrease in the cell count of bacterially injected Galleria mellonella larvae which was accompanied by bacterial clearance. These findings indicated that the overall decline in the THC may be due to the phagocytic cell population dropping out of circulation. This selective depletion of plasmatocytes was suggested by Chain and Anderson (1982; 1983a) to be induced by a haemocytic factor released in response to infection. In a more recent study, Geng and Dunn (1989) found plasmatocyte depletion in the tobacco hornworm to be dependent on bacterial dose and induced specifically in response to bacterial challenge rather than wounding. The THC and proportion of plasmatocytes

in Locusta migratoria also decreases in response to inert particulate material (Brehelin and Hoffman, 1980). However, selective cell depletion is not peculiar to the plasmatocytes since Rizki and Rizki (1984) noted selective destruction of the lamellocytes of Drosophila melanogaster during infection with the parasitoid wasp, Leptopilina heteroma.

In the present work, after mock infection with latex spheres, the THC of cockroaches halved within 30min. Since the phagocytic plasmatocyte subpopulation represents only approximately 10% of the plasmatocytes under these conditions, this massive reduction in the THC is not due to loaded phagocytic cells dropping out of circulation. Saline injection alone causes a drop of 20% in the THC which implies that haemocyte depletion in Periplaneta is partly due to a non-specific wound response. Nodule formation occurs in response to infections in excess of the phagocytic capacity of the haemocytes (Ratcliffe and Walters, 1983) so the much lower THC in latex-injected compared to saline-injected animals is probably due to nodule formation stimulated by the particulate latex. However, latex beads adhered to all tissues of the gut so margination to the haemocoel lining or specific tissues could explain the rapid non-specific decline in the THC. The dorsal diaphragm of insects had far more adherent beads than other tissues although whether these were internalised is not known. Surprisingly, Brehelin and Hoffman (1980) never found latex beads in the pericardial cells of locusts, even though these cells readily phagocytosed iron saccharate.

Hymenolepis had a highly stimulatory effect on phagocytosis, even from within the gut. The 20% increase in the cell count 1 day after infection can be almost entirely attributed to phagocytic cells and the

day 3 results, in which both the THC and the percentage of phagocytic plasmatocytes have returned to control levels, support this suggestion.

These results show that changes in the haemogram and phagocytic capabilities are induced rapidly after infection and are indicative of an activated immune system. Although phagocytosis is unlikely to be of much practical use in the event of a large organism eg. a helminth entering the haemocoel, the immune system clearly responds with whatever means are available. The end-product is a highly responsive and competent immune system ready to launch into the next battle strategy, perhaps encapsulation, if phagocytosis and nodule formation are unsuccessful.

SUMMARY

1. Circulating cell number, nodule formation and phagocytosis have been used in this chapter to quantitatively assay the immune response of P. americana to Zs, H. diminuta and M. moniliformis.

The results presented in this chapter suggest that oncospheres of H. diminuta stimulate the immune system whereas acanthors of M. moniliformis depress some aspects of the immune response. In contrast, cockroaches harbouring established infections of stage II/III acanthellae showed no evidence for either immunostimulation or suppression.

2. Immune stimulation with Zs caused a short-lived decrease in the circulating cell number but had no effect on the phagocytic cell population. Nodule formation was induced by Zs injection.

3. Mature oncospheres of Hymenolepis induced an increase in the cell number and phagocytic uptake 1 day after feeding to cockroaches.

4. Nodules were formed in cockroaches fed Moniliformis acanthors and the THC was reduced in these animals.

5. Cockroaches experimentally infected with Moniliformis acanthellae had cell counts comparable with naive and control animals. There was no relationship between the cell count and survival of transplanted acanthellae.

6. The functional significance of the results for the THC and phagocytic uptake is discussed and suggestions have been made for the mechanism by which the parasites might stimulate the immune system.

CHAPTER 4 - ENCAPSULATION AND CELL SPREADING

CHAPTER 4 - ENCAPSULATION AND CELL SPREADING

INTRODUCTION

Cellular Encapsulation

Encapsulation, in which haemocytes adhere to and spread over the foreign material to form multilayered sheaths, is of prime importance in immunity of invertebrates to metazoan parasites. Immunorecognition and subsequent encapsulation of foreign bodies, both biotic and abiotic, is dependent on the surface properties of the material. Encapsulation by insect haemocytes has been studied using biotic material (Ennesser and Nappi, 1984; Lackie, 1979) but this presents complications because of the complex and undefined nature of the surface molecules and the evolution of metabolic by-products. As a result, abiotic particles eg. ion exchange beads, are frequently used for encapsulation assays because of their relatively well-defined and consistent surface properties (Dunphy and Nolan, 1980b; Lackie, 1983; Stoltz and Guzo, 1986). Other abiotic non-self targets which have been studied for capsule-promoting abilities include glass, nylon, epoxy resin (Salt, 1970) and araldite (Grimstone et al., 1967; Schmit and Ratcliffe, 1978), and styrene particles (Misko, 1972).

Capsule thickness corresponds with the degree of foreignness since Lackie (1979) found that grafts from closely related species remained unencapsulated or thinly encapsulated in contrast with the thick capsules formed around grafts from distantly related species. However, capsule thickness may also be expected to be a function of the number of circulating cells and the state of activation of the immune system.

In the previous chapter, it was shown that the immune system of the insect was affected by both of the parasites and the Zs, using circulating cell number and phagocytic capacity as indicators of immune activation. This information indicated overall changes in the immune reactivity but provided little information on specific aspects of cell behaviour, although the nodule assay suggested that alterations in cell adhesiveness may be an aspect of immune activation.

The aim of the current chapter was to investigate the behavioural changes, especially adhesion and spreading, in the cells of the immune system after stimulation. Capsule thickness *in vivo* and *in vitro* has been used as a measure of immune reactivity. These experiments tested the hypothesis that once the immune system has been activated by a specific biotic stimulant eg. *Hymenolepis*, then the response to an abiotic non-specific stimulant eg. Sepharose beads, may be enhanced.

The simple *in vitro* encapsulation assay used in this chapter provides the opportunity to investigate haemocyte adhesion under clearly defined conditions and several authors have adopted this experimental approach (Vinson, 1974; Lackie, 1983; Ratner and Vinson, 1983a; Persson *et al.*, 1987). The system facilitates experimentation, since several treatments can be administered to each cell sample, separated cell populations can be used (Persson *et al.*, 1987), solutions can be changed readily and the development of the capsule can be monitored continuously.

Preliminary experiments using the *in vitro* adhesion assay were carried out to define appropriate culture conditions for capsule formation by cockroach blood cells, whilst later experiments were conducted to investigate the importance of surface charge of the test particles in recognition and attachment by cockroach haemocytes. Finally the assay was used to investigate the influence of parasitic

infection on adhesion and encapsulation.

Cell Spreading

After adhering to non-self material, haemocytes spread to form the multilayered, interdigitating sheaths typical of capsules (Grimstone et al., 1967; Ratcliffe and Gagen, 1977). Therefore, disruption of the stages of capsule formation could reduce the immunocompetence of the host, for example, if cell spreading was affected by the parasite.

Examples of parasites inhibiting cell spreading exist in the literature and several authors have found altered capsule formation in parasitised hosts. Davies, Strand and Vinson (1987) reported that haemocytes of Heliothis virescens treated with calyx fluid from the parasitoid, Campoletis sonorensis spread more slowly than controls. In an earlier study, Stolz and Guzo (1986) found similar effects on the spreading and encapsulation by haemocytes of Malacosoma disstria either infected with the parasitoid wasp, Hyposter fugitivus or injected with purified polynavirus. Haemocytes from Biomphalaria glabrata infected with Echinostoma paraensei are reported to be smaller and less able to adhere to a foreign surface (Noda and Loker, 1989).

Filopodial elongation is disrupted in haemocytes of the common armyworm, Pseudaletia separata infected with the parasitoid, Microplitis mediator and the ability of these hosts to encapsulate Sepharose beads is also reduced (Tanaka, 1987a). Wago (1983) proposed that the extent of filopodial elongation may determine the phagocytic capacity of haemocytes and Davies and Preston (1987) demonstrated a link between the spreading of insect haemocytes and immune function. These authors found that an intact microfilament system was required

for cell spreading and successful encapsulation reactions *in vitro*.

The aim was to find whether changes in the spreading or morphology of cells on defined substrata *in vitro* could be detected after treatment *in vivo*. The hypothesis was that enhanced spreading may indicate increased immunocompetence. Alternatively, if the haemocytes were unable to spread then the cellular immune response would be depressed because of diminished capsule formation.

Information gained in this chapter on cell adhesion and spreading could shed some light on mechanisms of host resistance and parasite survival.

RESULTS

4.1. *IN VITRO* ENCAPSULATION

A variety of experiments were done to define the basic experimental system and the most suitable culture conditions were used in later experiments.

4.1.1. Cell Number

Plate 4.1.1. shows encapsulated beads.

Encapsulation occurred when $0.5x10^6$ or more cells were included in the culture vial with the positively charged ion exchange beads. At very low cell densities ($0.25x10^6$), cells adhered to the beads in irregular masses but most of the bead surface remained unencapsulated. Capsules became thicker and more continuous as cell number increased. However, coagulation of haemocytes not associated with capsules was greater as cell number increased and these coagulated cells were often lysed. Continuous agitation by rotation of the culture was required for capsule formation, as otherwise haemocytes settled to the bottom of the culture vial embedding the beads in coagulum. There was no change in the morphology of the capsules as cell density increased. The cells formed loose, flocculent capsules with an irregular outline, in contrast with the smooth, multilayered capsules formed *in vivo*. Adherent haemocytes could be seen at varying stages of flattening on the surface of the beads. The cells towards the outer surface of the capsules were usually more rounded and often appeared slightly disintegrated. Encapsulated beads were commonly found in aggregates of varying size.

4.1.2. Length of Time in Culture

The structure of capsules was similar whether the cells were cultured for 1hr or up to 3hr. After 3hr in culture the adherent cells had spread extensively over the surface of the bead. The capsule did not increase in thickness with a longer time in culture.

Using trypan blue exclusion to test for cell viability, at the start of the experiment 90% of cells were viable. After 1hr, 56% of the cells not incorporated into the capsules excluded the dye and this was further reduced to 47% by 3hr in culture suggesting that culture conditions may not be ideal for long-term culture.

4.1.3. The Characteristics of Encapsulating Haemocytes

See Plate 4.1.3. for results.

Plasmatocytes are the predominant cell in encapsulation reactions (Ratcliffe and Gagen, 1977). They are phagocytic, contain both acid phosphatase and peroxidase. Haemocytes adhering to the ion exchange beads contained both acid phosphatase and peroxidase. Sites of peroxidase activity were clearly granular, whereas the acid phosphatase activity was only occasionally granular and more often dispersed throughout the cytoplasm. Haemocytes which had phagocytosed latex spheres *in vivo* adhered to the positively charged Sepharose beads in the *in vitro* assay.

4.1.4. The Effect of the Surface Charge of Beads on Encapsulation

Plates 4.1.4.a-c show encapsulated beads.

Haemocytes did not adhere to Sepharose beads carrying a neutral charge. However, silk loops present in the vials were encapsulated,

indicating that the cells retained the ability to discriminate between different surfaces. Positively charged Sepharose beads were better encapsulated by haemocytes than negatively charged beads, although the morphology of the capsules was similar for both types of bead. These results are mostly in agreement with observations made by Lackie (1983) *in vivo* (see chapter Discussion), suggesting that the *in vitro* assay provides reliable results.

4.1.5. Ultrastructure of Adhering Haemocytes

See Plates 4.1.5.a-d for results.

After 1hr in culture, the cells adhering to positively charged Sepharose beads were in close apposition to the bead surface. These cells were granular to varying degrees and contained many mitochondria. Numerous filopodial extensions, typical of haemocytes in culture (Lackie *et al.*, 1985), covered the surface of the cells. Lackie and co-workers (1985) found that haemocytes flushed out with anticoagulant often contained vacuoles, yet vacuoles were not present in the haemocytes around the beads or in those remaining in culture indicating good culture conditions. Since the subpopulations of cells fixed in suspension cannot be distinguished, it is impossible to identify which cells are not incorporated into the capsules. However, it is most likely that the encapsulating cells are plasmatocytes because they have the capacity to spread on contact with the beads.

4.1.6. Infection with *H. diminuta*

Haemocytes from *Hymenolepis*-fed cockroaches did not adhere well to the Sepharose beads (see Plate 4.1.6). Those cells which did adhere had the typical spread plasmatocyte morphology, as in control vials.

4.2. IN VIVO ENCAPSULATION

Capsule thickness around abiotic particles was used to determine whether parasite survival or elimination could be attributed to changes in the cell populations involved in encapsulation.

4.2.1. Infection with H. diminuta

The results are presented in Tables/Figures 4.2.1.a-d.

In both naive and experimental animals, although the majority of Sepharose beads were encapsulated, some remained unencapsulated in a small number of cockroaches. These beads were occasionally melanised which aided in their identification; unmelanised and unencapsulated beads were particularly difficult to find in dissected cockroaches.

The structure of the 24hr capsule from naive animals is shown on Plate 4.2.1. and is similar to capsules described by Grimstone et al. (1967). Using phase contrast optics, the capsule can clearly be seen to be composed of at least two or three layers. The inner layers are composed of necrotic cells, often melanised and surrounded by concentric sheets of flattened cells. The outermost layer is composed of rounded cells.

Capsule thickness around beads recovered from individual animals show considerable variation. Similarly, the mean capsule thickness around beads varies significantly from animal to animal. One day (Fig. 4.2.1.a) after feeding with H. diminuta the capsule around the Sepharose beads was significantly thicker in replicate 1 ($H=7.824$, $d.f.=2$, $p<0.05$) and significantly thinner ($H=16.71$, $d.f.=2$, $p<0.01$) in replicate 2. This is possibly due to high variation.

There

was no marked change in the percentage of beads melanised after feeding. Three days (Fig. 4.2.1.b) after feeding, the capsules were thinner in both replicates (Exp 1 - $H=11.65$, d.f.=2, $p<0.01$; Exp 2 - $H=23.57$, d.f.=2, $p<0.01$) compared to naive animals . Beads from control animals fed immature Hymenolepis had thinner capsules and were more melanised than in naive animals, which is possibly due to a few mature oncospheres being present in the proglottid.

4.2.2. Infection with M. moniliformis

Figure 4.2.2. summarises the data in Table 4.2.2. M. moniliformis acanthellae in the haemocoel had no effect on the thickness of capsules around beads ($H=0.05$, d.f.=2, $p>0.05$) or the percentage of beads melanised. Almost all the stage II acanthellae had developed to stage III or IV during the 8 day culture period and the recovery rate was approximately 75-80% of injected parasites.

4.2.3. Enzymes in Encapsulating Haemocytes

The cells in the capsules contained both acid phosphatase and peroxidase and there was no differentiation in intensity of stain between the 2-3 layers of the capsule. The intensity of staining was similar for beads recovered from control and experimental animals.

4.3. CELL SPREADING

Most observations on haemocyte spreading are qualitative rather than quantitative. In the work reported below quantitative measurements of cell spreading have been made using a computerised image processor which assesses the total area of the spread cells.

These experiments are concerned with plasmatocyte spreading because the coagulocytes do not spread over the substratum, are limited in their adhesion, and often lyse in vitro. Haemocytes were spindle shaped in suspension and underwent marked morphological changes on attachment to the substratum. Within 15-20 mins plasmatocytes had spread extensively and constituted a large proportion (80-90%) of the adherent haemocytes. Although coagulocytes were observed during the incubation period these cells were lost after processing of coverslips. Davies and Preston (1985) have detailed the morphological changes in haemocytes from lepidopteran species during settling out of suspension, spreading and locomotion.

4.3.1. Adhesion to Protein-Coated Glass

The results are shown in Figures 4.3.1.a-b.

The aim of these experiments was to find a surface to which haemocytes would stick but on which they would not spread fully. Haemocytes spread well on glass coated with 10% heat-inactivated foetal calf serum (FCS) (see Fig. 4.3.1.a); their area approximating 90% of the spread area on glass. There were no significant differences in the mean spread area of cells from different experiments.

Spreading on bovine serum albumin (BSA)-coated glass was less than on FCS-coated glass. Cell adhesion was less on coverslips incubated with high (10mg/ml BSA) concentrations of protein compared with those incubated with less protein (0.1mg/ml BSA) (see Fig. 4.3.1.b). The mean spread area of cells was unaffected by protein concentration, except in Exp3, in which the spread area of cells was significantly smaller on 1mg/ml BSA compared with 0.1mg/ml BSA. However, Takle (1986) found that high BSA-binding to polystyrene reduced the spread area of

haemocytes. These conflicting results may be due to differences in cell handling or variations in the binding of BSA to glass as opposed to polystyrene or variability in the properties of different batches of BSA. There were significant differences between experiments, possibly due to variations in the *in vitro* culture conditions eg. binding of BSA to glass.

4.3.2. Zymosan A Supernatant Injection

Zs pre-treatment did not affect haemocyte spreading on glass coated with BSA compared to naive or HBS-injected controls (see Fig. 4.3.2).

4.3.3. Infection with *H. diminuta*

Infection with oncospheres of *Hymenolepis* did not alter the ability of cells to spread on protein-coated glass (see Fig.4.3.3.). There was no significant difference in the mean spread area of haemocytes from control or experimental animals on either 0.1 or 1mg/ml BSA in both replicates.

Plate 4.1.1

Phase contrast micrograph of positively-charged Sepharose beads encapsulated in the *in vitro* assay by cells from naive cockroaches. Scale bar=300μm. Note adhesion and spreading of plasmatocytes (PL) over bead surface (B).

Plate 4.1.6

Phase contrast micrograph of encapsulation of positively-charged Sepharose beads encapsulated *in vitro* by cells from *Hymenolepis*-infected cockroaches. Note fewer cells adhering to the bead compared with naive animals (Plate 4.1.1).
Scale bar = 300μm

Plate 4.1.1.

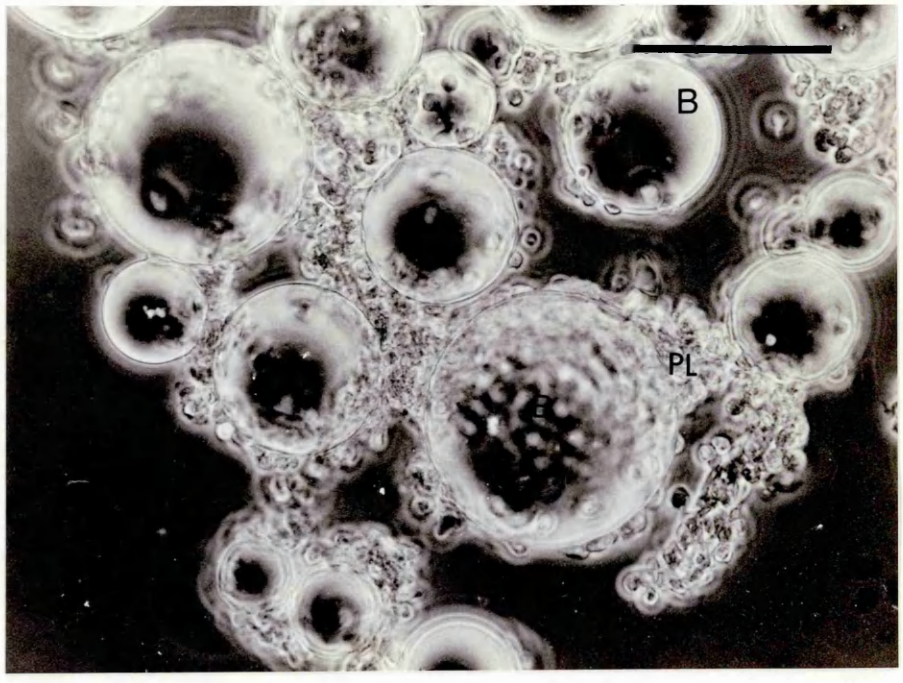
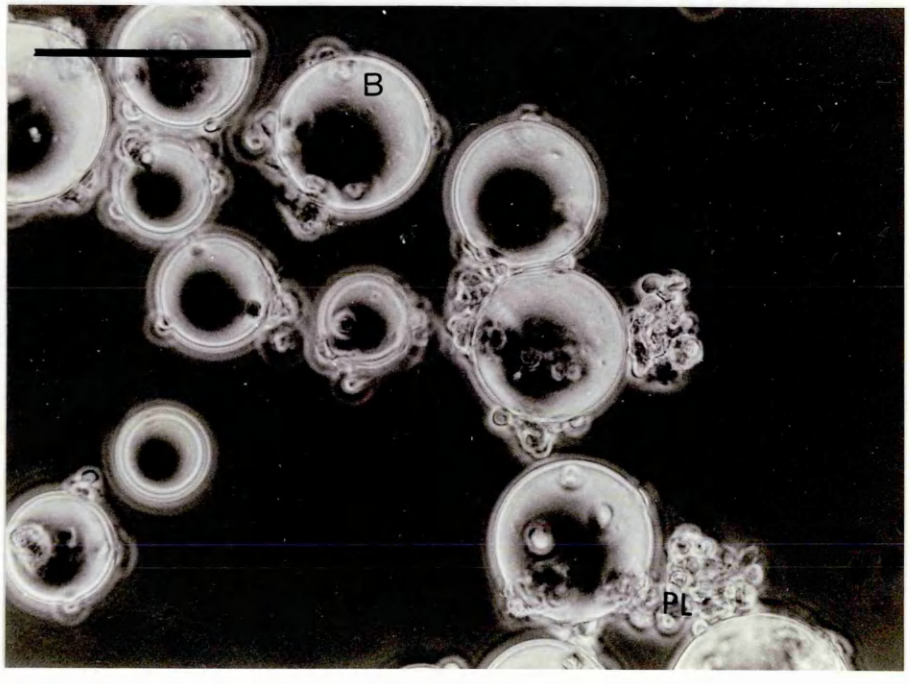


Plate 4.1.6.



Light micrographs of encapsulated Sepharose beads stained for intracellular enzymes.(B = BEAD)

Plates 4.1.3.a-b

a. Scale bar = 100μm. 1hr *in vitro* capsule containing dark brown peroxidase reaction product.

b. Scale bar = 100μm. 1hr *in vitro* capsule containing blue acid phosphatase reaction product.

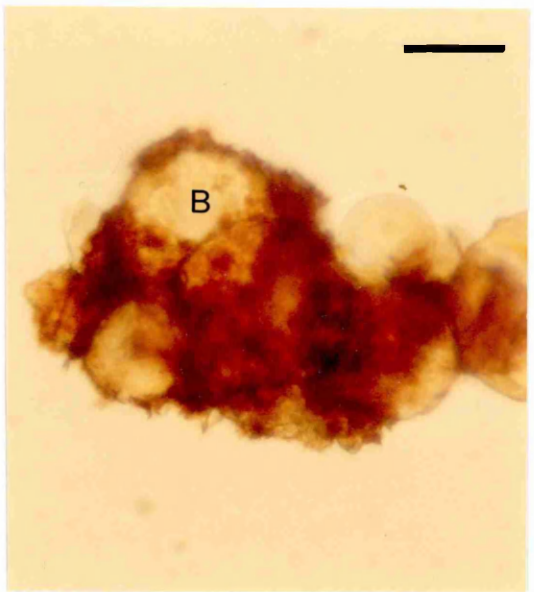
Plates 4.2.3.a-b

a. Scale bar = 100μm. 24hr *in vivo* capsule containing dark brown peroxidase reaction product.

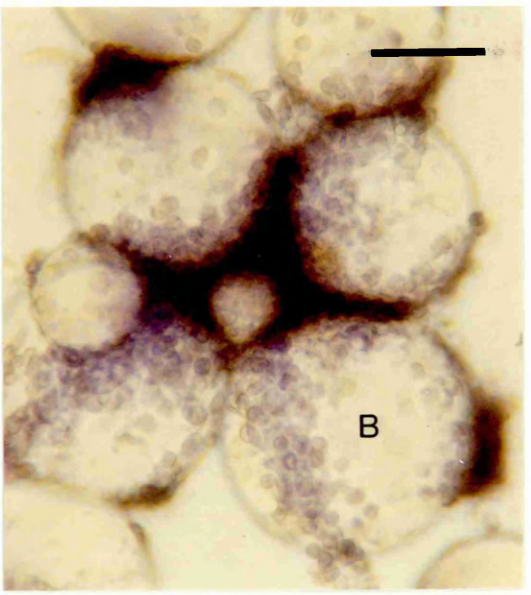
b. Scale bar = 100μm. 24hr *in vivo* capsule containing blue acid phosphatase reaction product. Note melanisation of inner capsule layers.(M).

Plates 4.1.3.a-b

a.Peroxidase

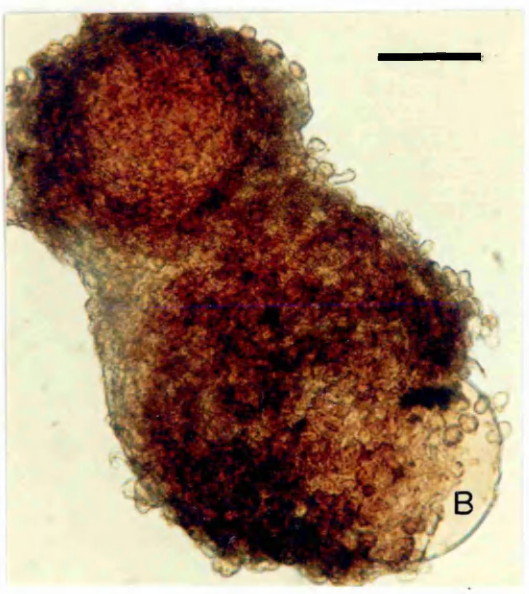


b.Acid Phosphatase

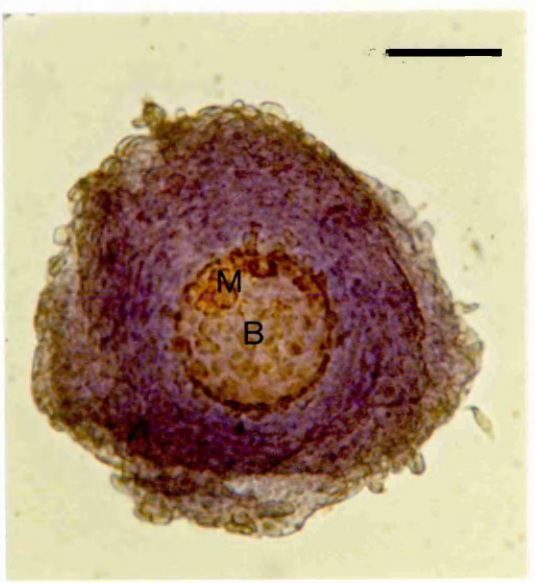


Plates 4.2.3.a-b

a.Peroxidase



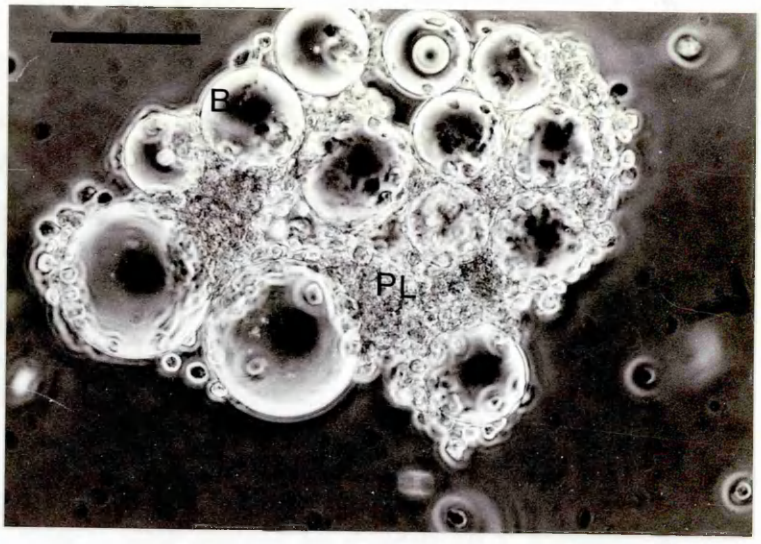
b.Acid Phosphatase



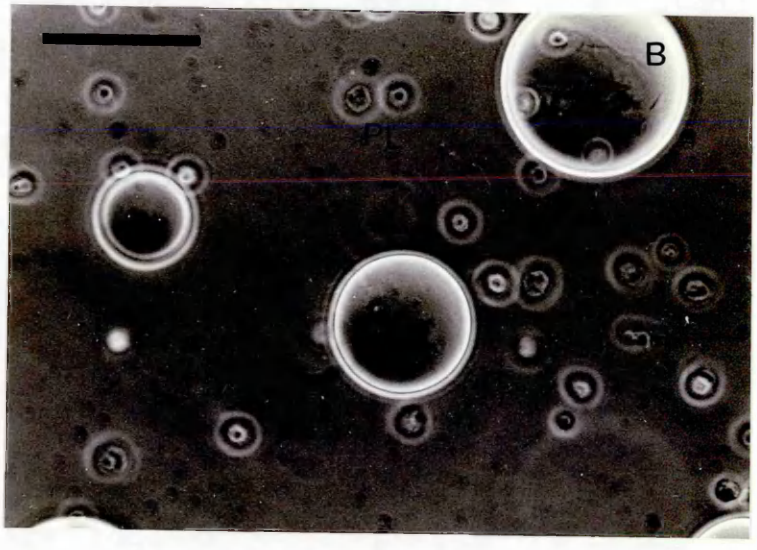
Plates 4.1.4.a-c

The effect of surface charge on encapsulation. Phase contrast of encapsulation of Sepharose beads in vitro by haemocytes from naive cockroaches. Scale bar = 200μm. (B= BEAD, PL= PLASMATOCYTE)

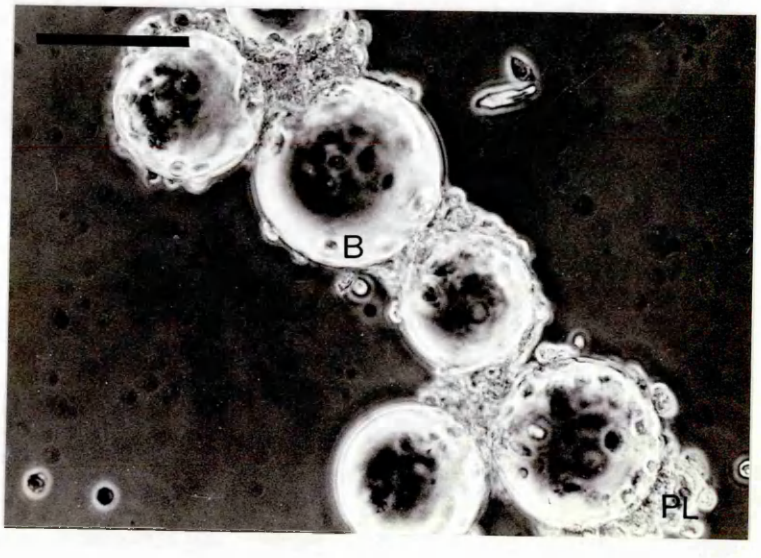
A. Positive



B. Neutral



C. Negative



Plates 4.1.5.a-d

Electron micrographs of positively charged Sepharose beads encapsulated *in vitro*.

a. 1hr incubation. Haemocytes are closely adherent to bead surface (B). Plasmatocytes have numerous cytoplasmic extensions (arrowheads) and folding. Many granules (arrows) are present within the cytoplasm. Mitochondria (M) present throughout the cytoplasm. Scale bar = 1µm

b. Cell contains fewer granules than above. Scale bar = 1µm

c. and d. Non-adherent haemocytes after 1hr in encapsulation assay. The cells are in good condition - few vacuoles or membrane blebbing. Note cytoplasmic extensions and numerous granules. Scale bar = 1µm

Plate 4.1.5.a.

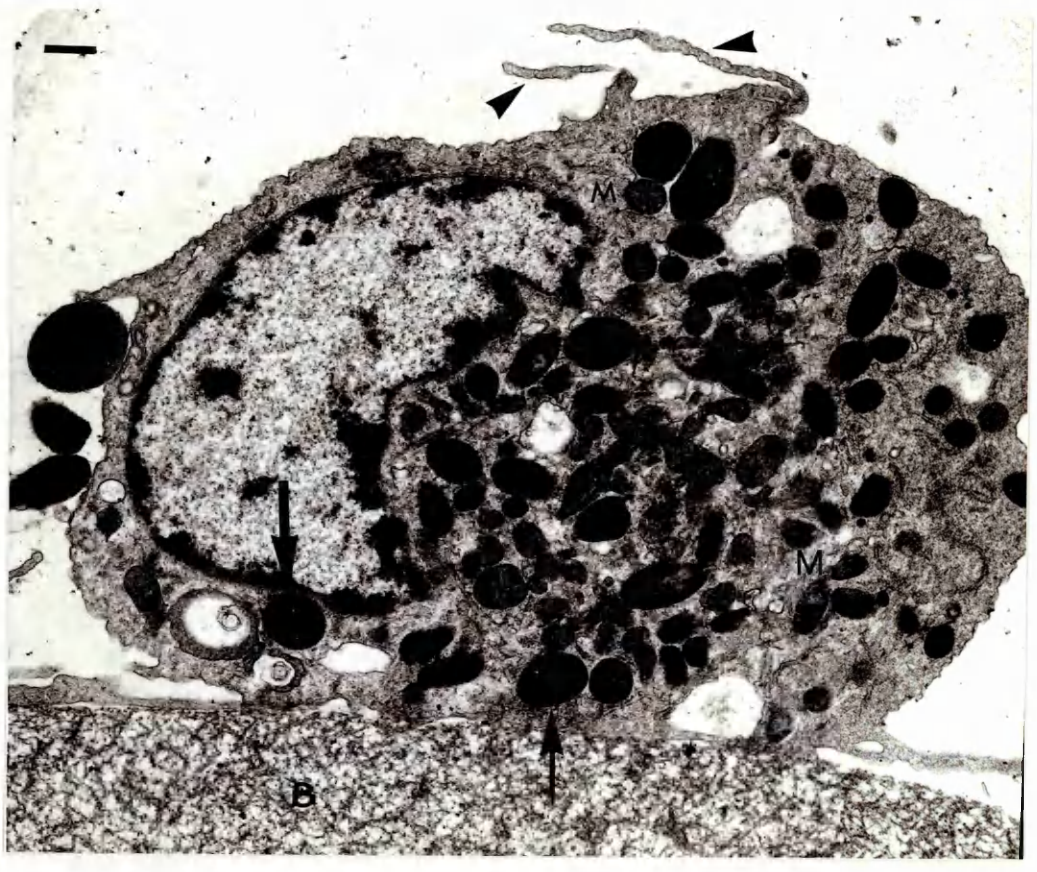


Plate 4.1.5.b.

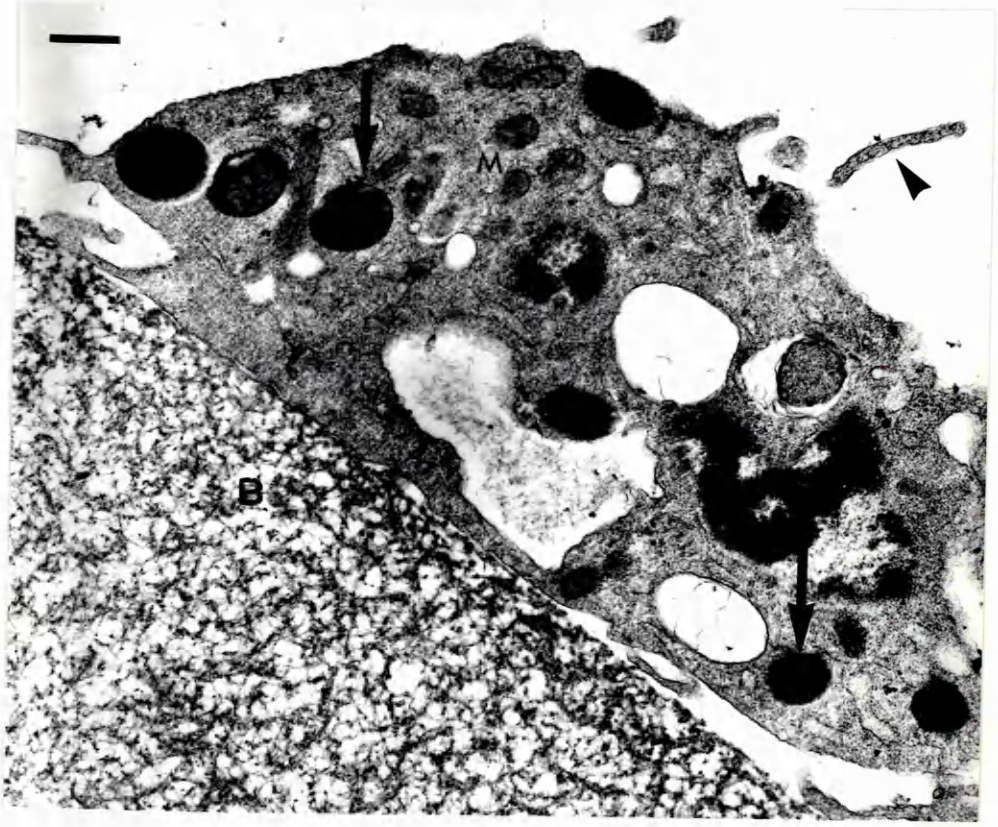


Plate 4.1.5.c.

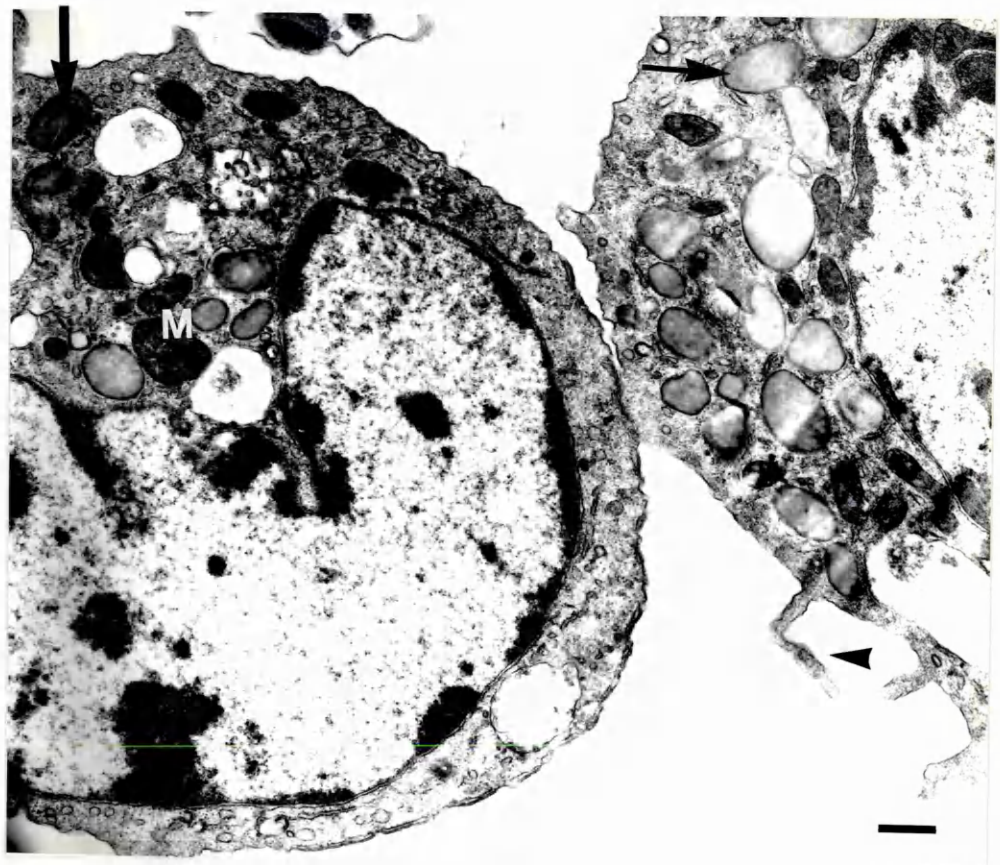
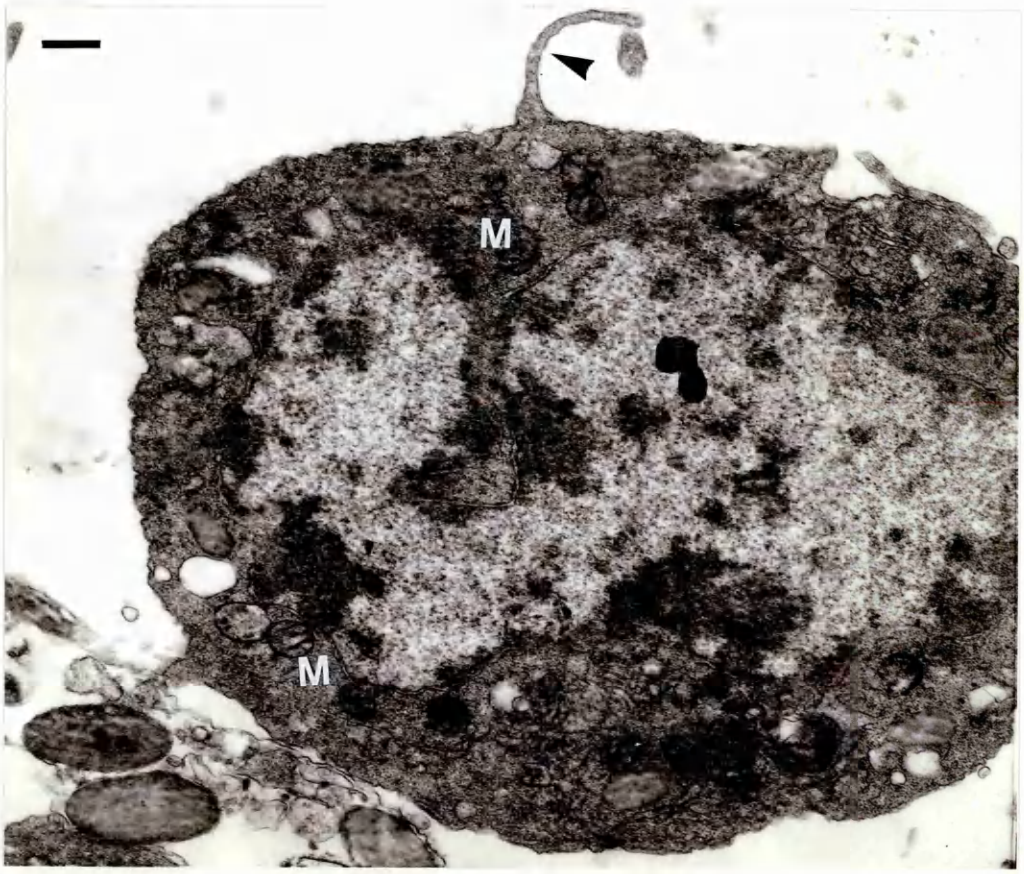


Plate 4.1.5.d.



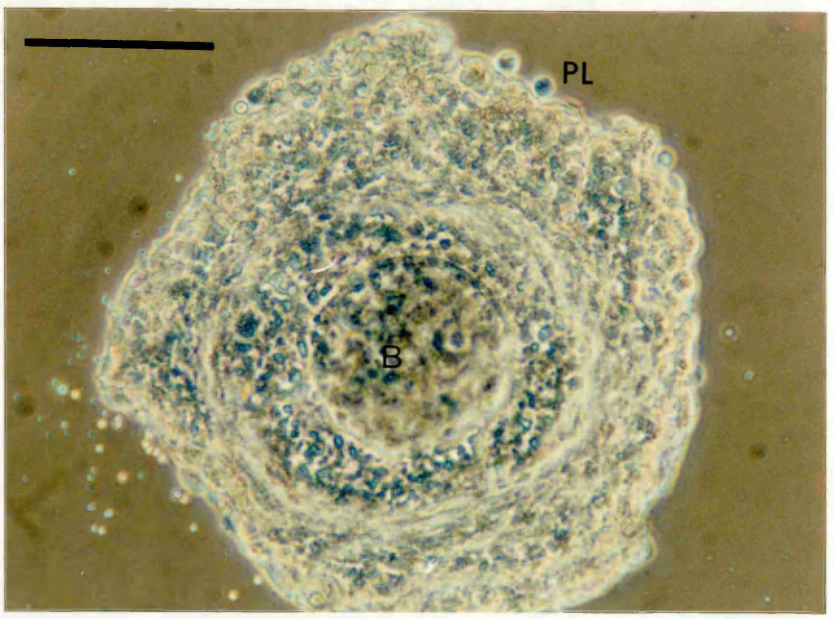
Plates 4.2.a-b

Phase contrast micrographs of negatively-charged Sepharose beads encapsulated by cockroach haemocytes in vivo (24hr capsule). (PL = PLASMATOCYTE)

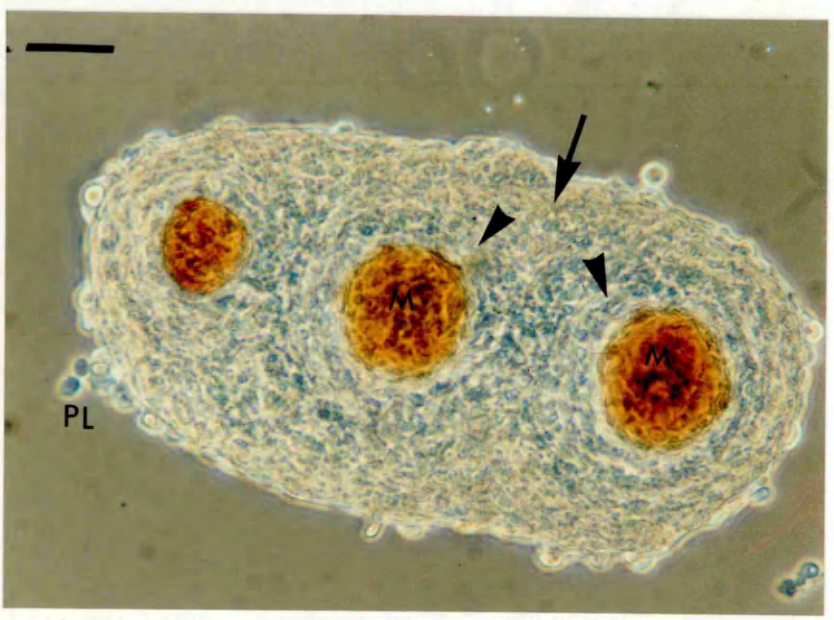
a. Encapsulated bead (B). Note plasmatocytes loosely attached at outer edge of capsule and inner compact layers of cells.

b. Capsule containing 3 beads. Note inner capsule formation around individual beads (arrowheads) and outer layer (arrows) which ensheaths all beads. Bead surface shows melanisation (M).

4.2.a. Scale bar = 200μm



4.2.b. Scale bar = 100μm



CAPSULE THICKNESS 1 DAY AFTER FEEDING WITH *HYMENOLEPIS DIMINUTA*

Table 4.2.1.a - Experiment 1

Encapsulation of CM-Sepharose (negatively charged) beads *in vivo* by haemocytes of adult male cockroaches fed *Hymenolepis* (*Hd*) 1 day previously. The capsule thickness was measured at 4 points around the bead and a mean value was calculated for each of the beads recovered from each animal. Data from individual animals were fused to obtain an overall mean for each treatment. Control animals were either naive or fed immature proglottids.
Imm = immature, Mat = mature

Treatment	Animal No.	No. Beads	Capsule Thickness (μm+s.d.)	% melanised
Naive	1	20	30.0+10.1	40
	2	8	50.1+17.1	12.5
	3	6	60.6+21.3	83
	4	20	44.3+12.6	45
	mean+sd	54	41.7+16.7	45.1
Imm *Hd*	1	16	38.1+28.4	68.8
	2	20	38.1+20.7	70
	3	8	43.1+16.3	0
	4	20	36.2+17.5	5
	5	13	47.2+10.8	14.3
	6	20	54.8+21.8	70
	7	7	41.6+14.5	14.3
	mean+sd	99	42.4+20.7	34.6
Mat *Hd*	1	20	43.9+12.2	85
	2	20	51.6+11.7	60
	3	20	38.6+9.5	30
	4	16	42.8+8.7	0
	5	20	50.1+24.5	15
	6	12	65.5+22.8	36.4
	mean+sd	108	47.5+17.1	37.7

CAPSULE THICKNESS 1 DAY AFTER FEEDING WITH *HYMENOLEPIS DIMINUTA*

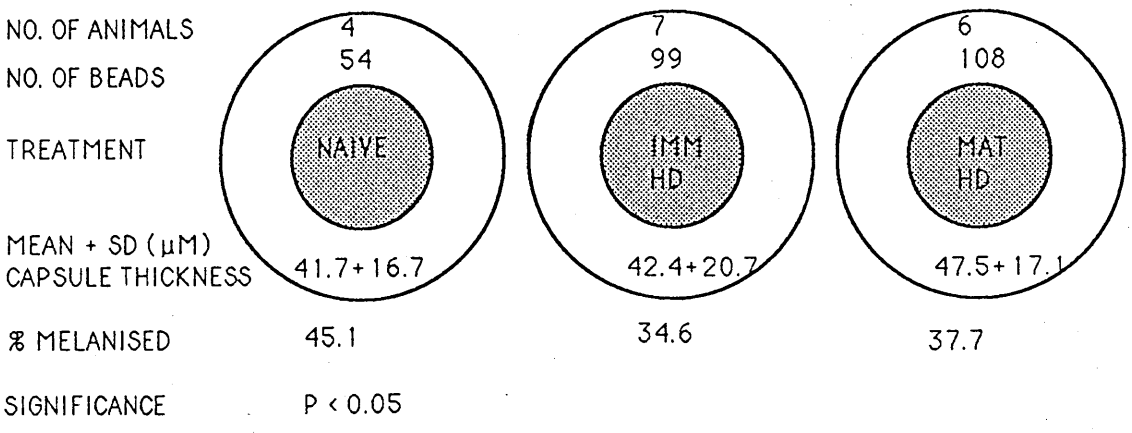
Table 4.2.1.a - Experiment 2

Encapsulation of CM-Sepharose (negatively charged) beads *in vivo* by haemocytes of adult male cockroaches fed *Hymenolepis* (Hd) 1 day previously. The capsule thickness was measured at 4 points around the bead and a mean was calculated for the beads recovered from each animal. Data from individual animals were fused to obtain an overall mean for each treatment.
Control animals were either naive or fed immature proglottids.

Treatment	Animal No.	No. Beads	Capsule Thickness (μm+s.d.)	% Melanised
Naive	1	21	52.8+18.3	75
	2	21	59.5+22.0	40
	3	20	50.7+30.9	15
	4	20	44.7+10.6	45
	5	20	29.8+10.3	20
	6	20	49.0+18.2	20
	7	20	80.2+33.6	25
	mean+sd	142	52.4+25.9	34.5
Imm *Hd*	1	20	40.8+17.8	25
	2	20	77.4+24.2	90
	3	20	60.9+22.6	5
	4	20	47.1+17.3	35
	5	20	60.2+18.9	50
	6	20	54.4+23.7	20
	7	20	41.7+10.9	75
	mean+sd	140	54.7+22.8	42.1
Mat *Hd*	1	20	31.4+9.6	35
	2	20	40.2+18.3	35
	3	20	40.5+12.7	35
	4	20	42.1+9.7	20
	5	20	63.6+26.7	55
	6	20	46.7+13.5	35
	mean+sd	120	44.1+18.7	35.8

CAPSULE THICKNESS 1DAY AFTER FEEDING WITH HYMENOLEPIS DIMINUTA

EXPERIMENT 1



EXPERIMENT 2

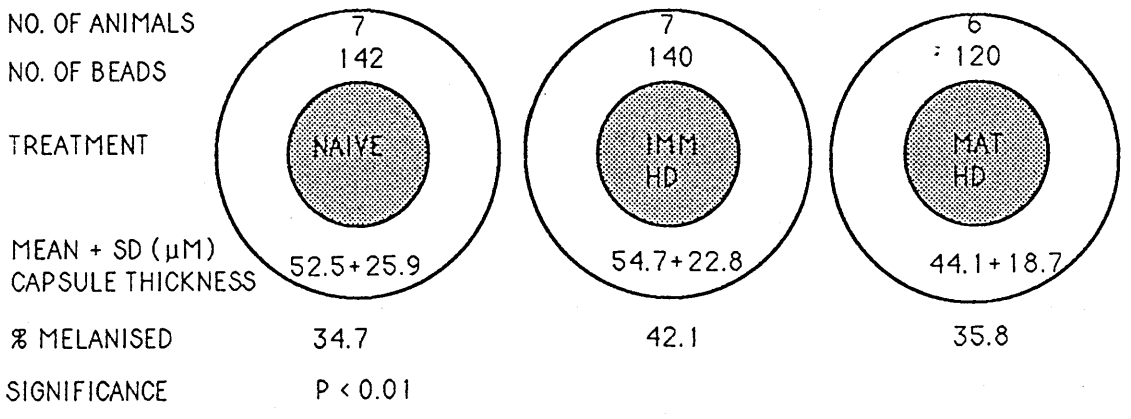


Figure 4.2.1.a
Capsule thickness (µm) around CM-Sepharose (negatively charged) beads encapsulated in vivo by haemocytes from cockroaches fed Hymenolepis diminuta 1 day previously. Significance tested using Kruskal-Wallis rank sum test.

CAPSULE THICKNESS 3 DAYS AFTER FEEDING WITH *HYMENOLEPIS DIMINUTA*

Table 4.2.1.b

Encapsulation of CM-Sepharose (negatively charged) beads *in vivo* by haemocytes of adult male cockroaches fed *Hymenolepis* (*Hd.*) 3 days previously. The capsule thickness was measured at 4 points around the bead and a mean value was calculated for each of 20 beads recovered from each animal. Data from individual animals were fused to obtain an overall mean for each treatment. Control animals were either naive or fed immature proglottids.
Imm. = immature, Mat. = mature.

Treatment	Animal No.	No. Beads	Capsule Thickness (μm+s.d.)	% Melanised
EXPERIMENT 1				
Naive	1	19	86.7+17.7	47.4
	2	20	49.5+19.2	10
	3	20	45.0+15.0	30
	4	20	39.0+14.1	40
	5	20	29.1+8.7	10
	6	20	36.3+14.7	50
	7	20	86.7+27.3	75
	mean+sd	139	53.1+27.9	37.4
Imm. *Hd*	1	20	30.0+10.2	60
	2	20	48.0+20.7	10
	3	20	34.8+15.6	10
	4	20	47.4+12.3	45
	5	17	40.5+16.2	5
	6	20	31.2+10.8	0
	mean+sd	117	38.7+16.2	21.8
Mat. *Hd*	1	20	49.2+16.2	35
	2	20	30.6+9.3	80
	3	20	30.0+8.4	5
	4	20	47.1+18.0	60
	5	20	38.7+14.4	5
	6	20	34.8+12.3	10
	mean+sd	120	38.4+14.7	32.5
EXPERIMENT 2				
Naive	mean+sd	10	69.1+27.9	50
Imm. *Hd*	mean+sd	41	46.5+27.9	78
Mat. *Hd*	mean+sd	16	33.2+13.8	100

CAPSULE THICKNESS 3 DAYS AFTER FEEDING WITH HYMENOLEPIS DIMINUTA

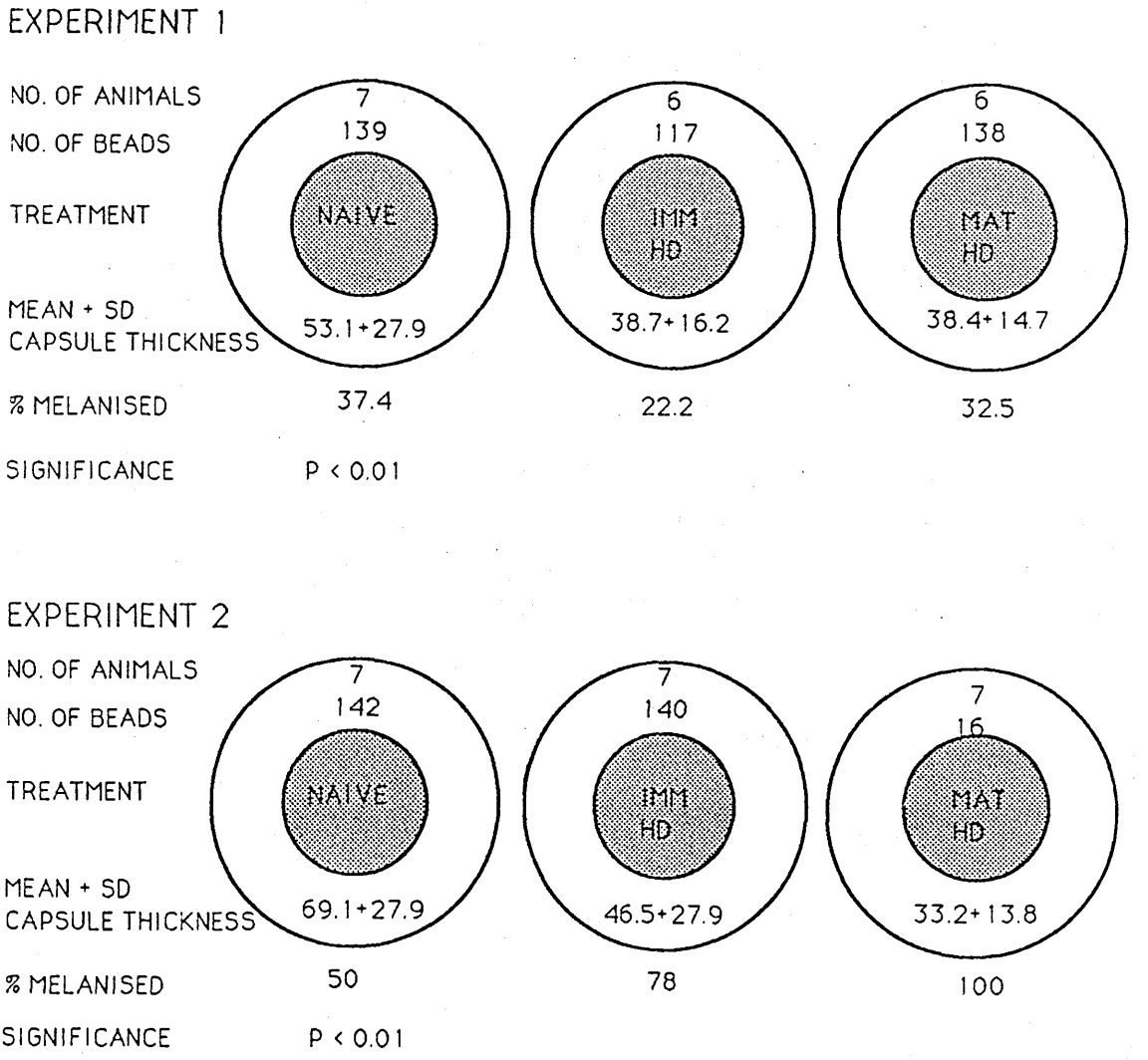


Figure 4.2.1.b
Capsule thickness (μm) around CM-Sepharose (negatively charged) beads encapsulated in vivo by haemocytes from cockroaches fed Hymenolepis diminuta 3 days previously. Significance tested using Kruskal-Wallis rank sum test.

CAPSULE THICKNESS AFTER INFECTION WITH MONILIFORMIS ACANTHELLAE

Table 4.2.2.

Encapsulation of CM-Sepharose (negatively charged) beads in vivo by haemocytes of adult male cockroaches infected with 40 acanthellae of Moniliformis 8 days previously. The capsule thickness was measured at 4 points around the bead and a mean value was calculated for each of 20 beads recovered from each animal. Data from individual animals were fused to obtain an overall mean for each treatment.
Control animals were either naive or injected with medium.

Treatment	Animal No.	No. Beads	Capsule Thickness (μm+s.d.)	% Melanised
Naive	1	5	44.8+8.5	60
	2	13	24.1+4.2	61.5
	3	6	36.2+19.3	50
	4	4	13.8+1.7	75
	mean+sd		28.9+13.8	60.4
Medium	1	7	27.5+13.2	42.8
	2	15	22.3+6.1	73.3
	3	4	39.6+6.8	50
	4	14	30.4+8.4	14.3
	5	10	23.0+8.1	30
	mean+sd		26.8+9.6	42.1
Moniliformis	1	4	31.4+8.2	50
	2	6	30.1+2.9	66.7
	3	4	43.5+10.3	75
	4	3	44.2+30.3	66.7
	5	14	22.0+6.5	92.9
	6	16	28.0+15.2	100
	mean+sd		28.9+13.5	75.2

CAPSULE THICKNESS AFTER INFECTION WITH MONILIFORMIS ACANTHELLAE

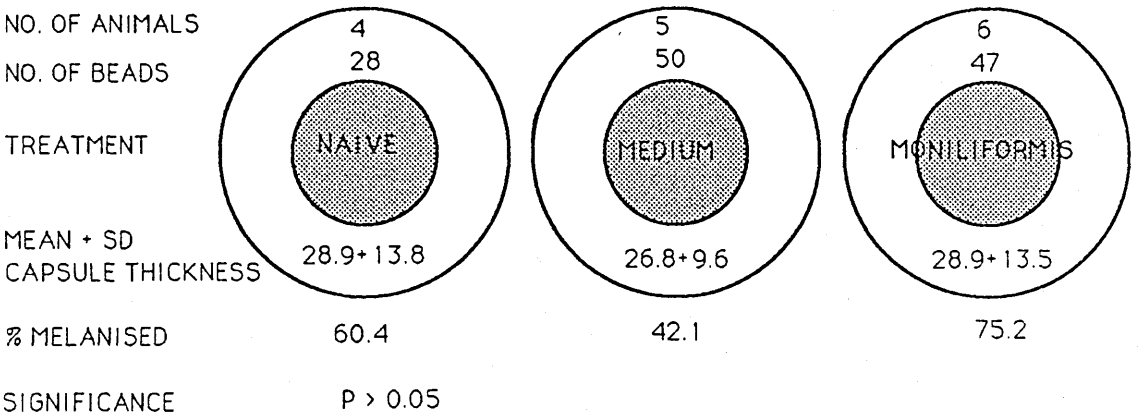


Figure 4.2.2.
Capsule thickness (μm) around CM-Sepharose (negatively charged) beads encapsulated in vivo by haemocytes from cockroaches infected with acanthellae of Moniliformis moniliformis.
Significance tested using Kruskal-Wallis rank sum test.

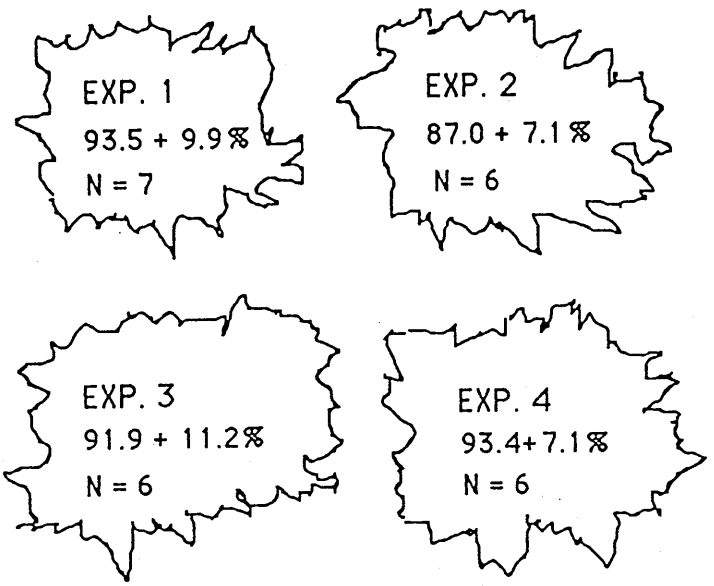


Figure 4.3.1.a
The spreading of haemocytes from naive cockroaches on glass coated with 10% heat-inactivated foetal calf serum in cockroach medium (D73). Results are expressed as a proportion (mean % + s.d.) of the spread area of haemocytes on glass which was normalised to 100%. Data are based on examination of > 100 cells / animal. N=number of animals.
All values non-significant using Mann-Whitney two sample rank test.

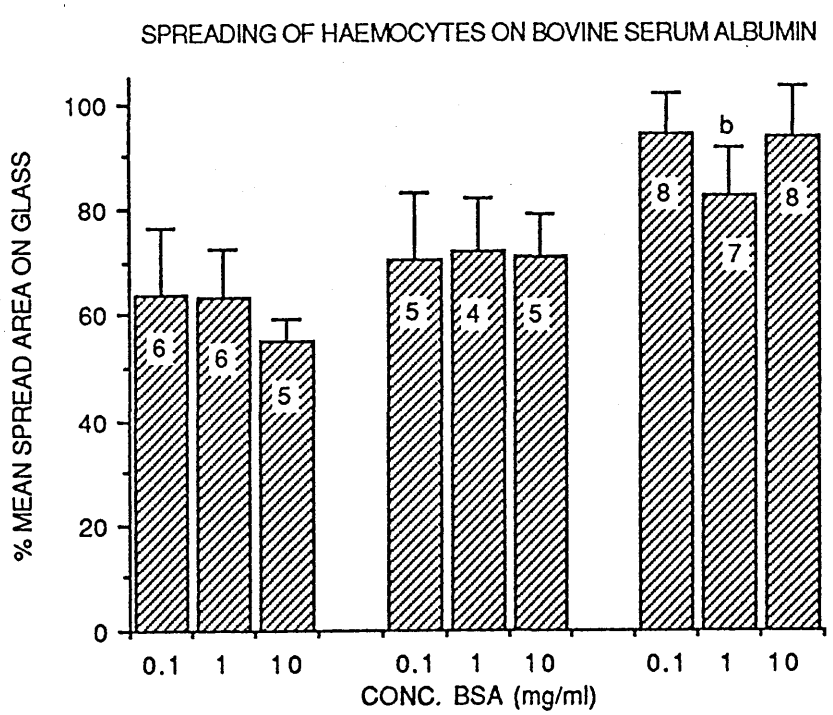
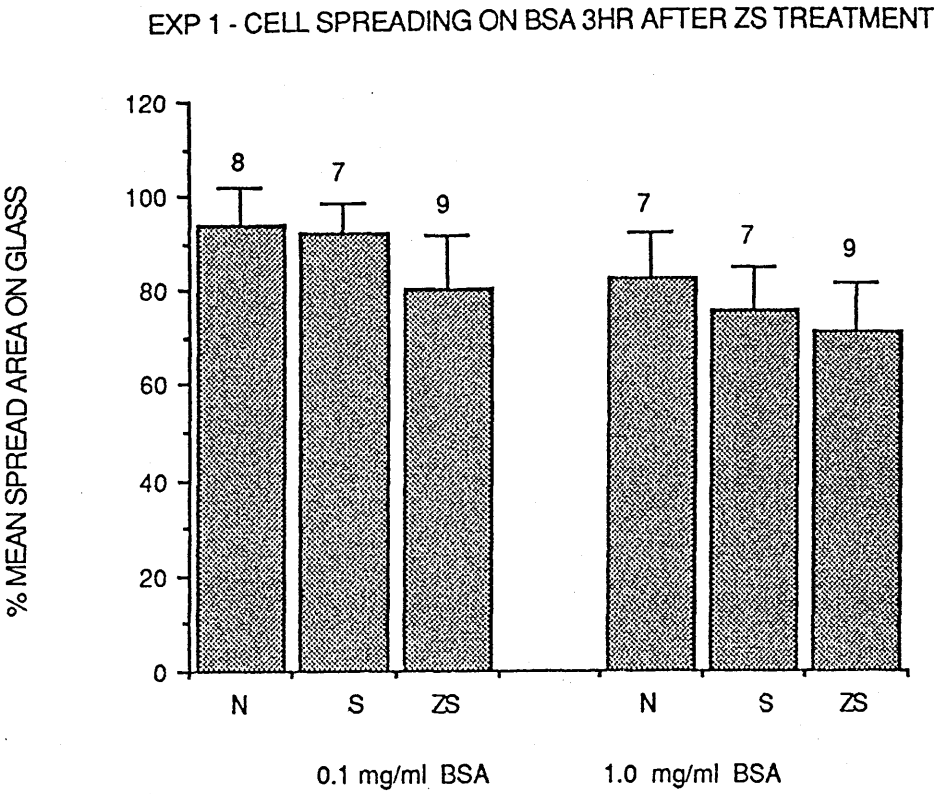


Figure 4.3.1.b
Spreading of haemocytes from adult male Periplaneta americana on bovine serum albumin (BSA)-coated glass. Results are expressed as a proportion (mean %+s.d.) of the spread area of haemocytes on clean glass which was normalised to 100%. Data are based on examination of > 100 cells / animal. Number of animals indicated under error bars.
b=$p<0.05$ compared with naive control using Mann-Whitney two sample rank test.



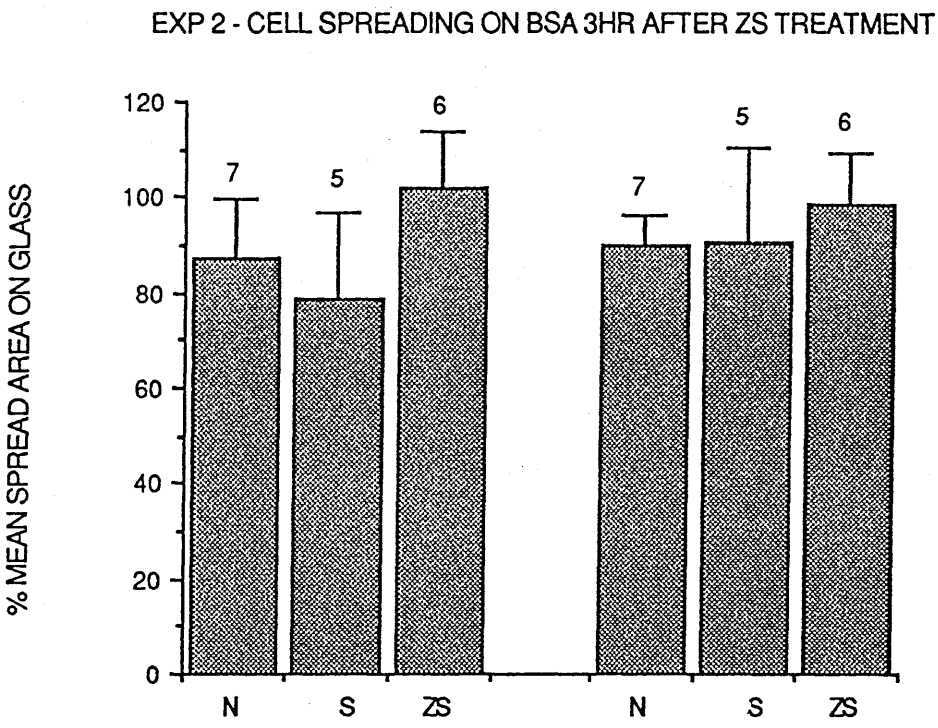
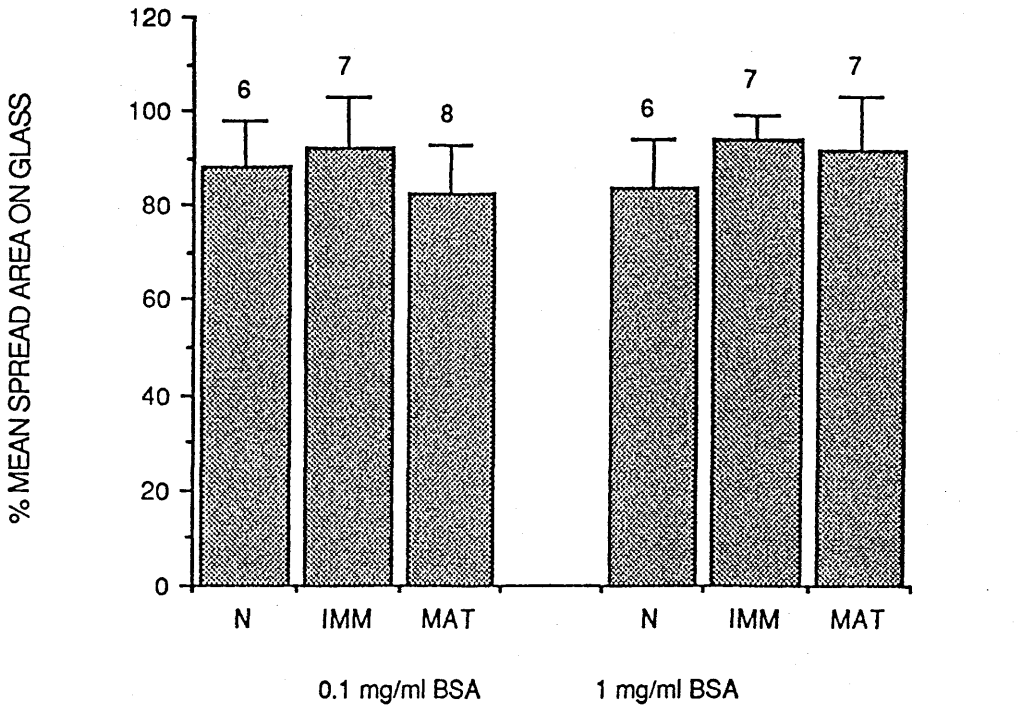


Figure 4.3.2.
Spreading on bovine serum albumin (BSA)-coated glass by haemocytes from cockroaches 3hr after injection with Zymosan A supernatant. Results are expressed as a proportion (mean % + s.d.) of the spread area of haemocytes on glass which was normalised to 100%. Data are based on examination of > 100 cells / animal. Number of animals indicated above error bars.
N = naive, S = saline, Zs = Zymosan A supernatant .
All values non-significant using Mann-Whitney two sample rank test.



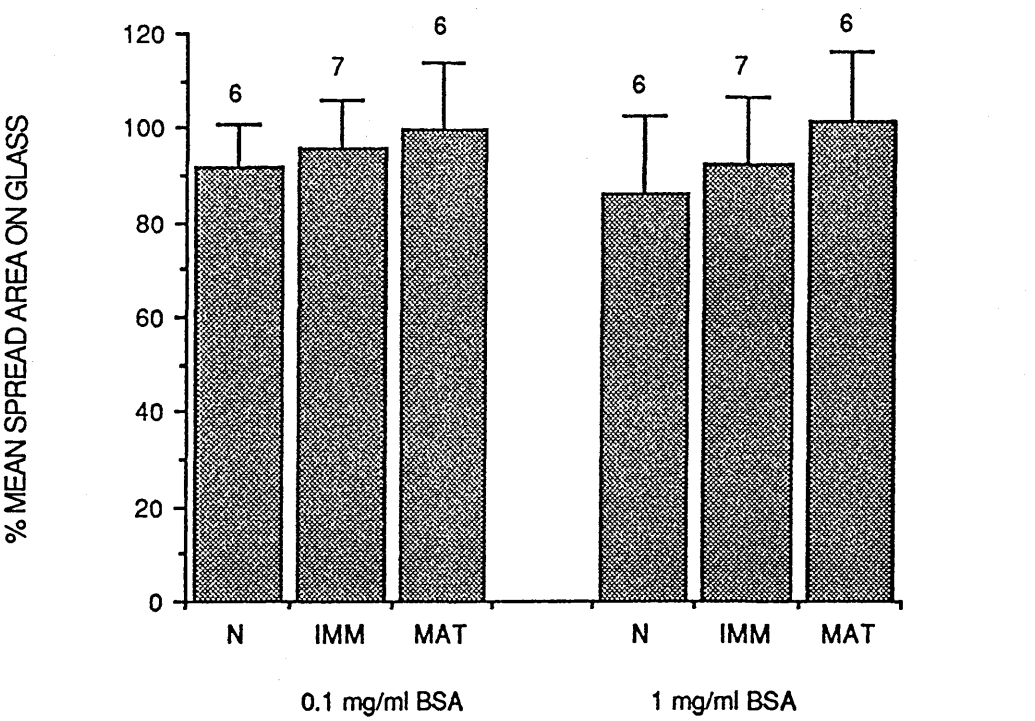


Figure 4.3.3.
Spreading on bovine serum albumin (BSA)-coated glass by haemocytes from cockroaches 1 day after feeding with Hymenolepis diminuta . Results are expressed as a proportion (mean %+ s.d.) of the spread area of haemocytes on clean glass which was normalised to 100%. Data are based on examination of > 100 cells / animal. Number of animals indicated above error bars.
N = naive, I = immature Hymenolepis, M = mature Hymenolepis.
All values non-significant using Mann-Whitney two sample rank test.

DISCUSSION

The thickness of capsules formed around target particles *in vivo* is highly variable depending on the nature of the particle (Lackie, 1983; Takle, 1986) and the ability of the immune system to recognise the non-self material. This variability in capsule thickness has been exploited in the experiments reported here to investigate the immune response. If the degree of encapsulation of a foreign object is related to the extent of immunostimulation or suppression, then information can be gained on the acuity of recognition and responsiveness of the immune system after infection by measuring the capsule thickness. Therefore, an abiotic particle injected into a pre-stimulated immune system is expected to elicit a thicker capsule than a similar particle in a suppressed immune system.

The plasmatocytes of *P. americana*, the main circulating cell type, is also the major capsule forming cell (Lackie *et al.*, 1985), although other cell types such as granulocytes (Ennesser and Nappi, 1984) are thought to be involved in various stages of capsule formation. Therefore, depletion or expansion of the plasmatocyte population by a stimulant is likely to have a profound effect on encapsulation. Several authors have used an *in vitro* encapsulation system to investigate parasitism in insects. Davies and Vinson (1986) discovered that host cells were incapable of encapsulating braconid wasp eggs and Dunphy and Nolan (1980b) found that the fungal pathogen, *Entomophthora egressa* remained unencapsulated after incubation with *Lambdina fiscellaria* haemocytes.

In the work reported here, the haemocytes in the *in vitro* assay not only retained the ability to discriminate self from non-self but could

also differentiate between the surface charge of the non-self material. This has important implications for the future use of this assay in investigations of the cockroach immune response. The adherent cells are more closely apposed to the bead surface than those recovered from the early stages of *in vivo* encapsulation (Lackie *et al.*, 1985) and this may reflect some stimulation of the cells by collection and handling *in vitro*.

Using the *in vitro* encapsulation assay the haemocytes of *P. americana* adhered well to positively charged Sepharose beads, less well to negatively charged beads and not at all to neutral beads. These results are in agreement with those of Lackie (1983) from studies of encapsulation of these beads *in vivo*. Cationised ferritin binding and cell electrophoresis studies (Takle and Lackie, 1985) indicated that cockroach haemocytes carried a net negative surface charge. Thus, on the basis of charge repulsion effects, it is anticipated that haemocytes would not encapsulate negatively charged surfaces. However, cell adhesion is more complex than simple charge repulsion. For example, haemolymph proteins may adsorb to the negatively charged surface so altering the surface properties of the bead. Therefore, in reality haemocytes do encapsulate negatively charged surfaces such as Sepharose beads and parasites.

Since *Hymenolepis* stimulates the cellular defence reaction of cockroaches (Holt, 1989a; Chapter 3) the encapsulation response to abiotic particles was studied. Surprisingly, the results show that the ability to form capsules is poorer both *in vivo* and *in vitro* in *Hymenolepis* infected animals. While the THC and phagocytic capacity of the cells appear to be enhanced by *Hymenolepis* infection, it seems these cells are not used in the encapsulation reaction. A possible

explanation is that haemocytes recruited from resting sites are immature and, therefore, not fully competent. However, if the ability to spread and phagocytose latex spheres is a measure of immunocompetence, then this explanation is unacceptable since haemocytes from Hymenolepis-infected animals respond well. By assessing the ability of haemocytes to spread after infection with Hymenolepis it is clear that the thinner capsules are not caused by the cells flattening more readily on the foreign surface. This is puzzling and requires further investigation into the fate of the cells released into the haemocoel on stimulation.

The thickness of capsules around the negatively charged ion exchange beads is unaffected by Moniliformis acanthellae in the haemocoel, in accordance with the results of Lackie (unpubl. obs.). This shows quite clearly that this component of the immune response, which requires recognition and competence to respond, is not compromised in Moniliformis infections. Whether Moniliformis larvae avoid encapsulation by presenting a negatively charged surface is unknown, but in the light of the preceding evidence, this seems to be unlikely since haemocytes are obviously capable of adhering to negatively charged surfaces. Christensen and his co-workers (1987) have found that microfilariae of Brugia pahangi lose their electronegativity during penetration of the mosquito midgut and they suggest that these changes are related to immune evasion.

Capsules were originally thought to kill the foreign material by preventing the passage of nutrients and oxygen to the organism (Salt, 1970). This is now generally accepted as incorrect, yet little has been provided by way of evidence for alternative killing mechanisms. The

prophenoloxidase pathway, which brings about the melanisation reaction frequently seen at the core of capsules, has been proposed as being involved in killing (Poinar et al, 1979). Quinones produced during the tanning process are probably toxic although other enzymes, such as the lysosomal enzymes, may also play a part in killing and digestion. Acid phosphatase and peroxidase have been visualised in capsules formed both in vivo and in vitro around Sepharose beads and the activity of both of these enzymes has been found in the pigmented capsules formed around nematodes by Periplaneta blood cells (Misko, 1972). Grimstone et al. (1967) have also located acid phosphatase activity in capsules around araldite implants in the insect, Ephestia. Since peroxidase is toxic to helminths in vertebrate systems (Jong et al., 1981) it may be involved in killing helminths in insects. Detailed discussion of these enzymes and their role in the defence reaction is presented in Chapter 6.

After haemocytes adhere around the target particle, they flatten; thus, immune stimulation may be accompanied by enhanced spreading. Indeed, adhesion and perhaps spreading, may be indicative of cell activation as in tumour cytotoxicity by vertebrate macrophages (Friedman and Beller, 1987).

Immune activation by pre-injection with Zs or infection with Hymenolepis did not enhance haemocyte spreading on a protein-coated substratum in vitro suggesting that in Periplaneta, haemocyte spread area may be influenced more by the properties of the substratum than by pre-stimulation. Furthermore, although capsule thickness may be a function of the extent to which cells spread, the number of haemocytes recruited into the multilayered sheath is more likely to influence capsule thickness.

Bovine serum albumin does not stimulate nodule formation (Lackie and

Vasta, 1986) in cockroaches in vivo therefore, it is a useful non-immunologic protein for use in these experiments. Takle (1986) found that adhesion and spreading of haemocytes on BSA-coated polystyrene was inversely proportional to the concentration of bound BSA. In the work reported here, although fewer cells attached to the BSA-coated glass at higher protein concentrations, the spreading was unaffected. These conflicting results may reflect differences in binding of BSA to glass compared with protein, or in the assessment of cell spreading.

Clean glass is highly surface active and the negatively charged cells of Periplaneta readily attach to and spread on clean glass. Reduced adhesivity of cells to BSA can be explained if this protein lowers the positive charge on the glass so that the effect of charge attraction between the cell and the substratum becomes less. Alternatively, cell adhesion to glass is mediated through molecules secreted by the cell to which the cells adhere and BSA blocks these cellular binding sites. At lower protein concentrations, BSA may not form a uniform covering in which case cell adhesions can form at glass foci. At higher protein concentrations few haemocytes can adhere and those which do have the ability to spread.

In summary, it seems that cell spreading is unaffected by any of the treatments administered to the insects prior to haemolymph collection. These results suggest that immune stimulation in the cockroach is not accompanied with an enhanced ability to spread over the foreign material.

SUMMARY

The immunocompetence of the immune system has been examined by investigating the ability of haemocytes to encapsulate ion exchange beads *in vivo* and *in vitro* and to spread on protein-coated glass.

1. Capsules formed *in vivo* and *in vitro* were usually significantly thinner after infection with *Hymenolepis*. This may be due to increased nodule formation after infection leaving fewer cells to respond to a second stimulus ie. Sepharose beads.

2. *Moniliformis* acanthellae had no effect on the thickness of capsules formed *in vivo* suggesting immune evasion rather than immune suppression by the parasite.

3. Substratum charge altered the ability of cells to adhere *in vitro*. Positively-charged beads were more readily encapsulated than negatively-charged beads and cells did not adhere at all to neutral beads. These results are in agreement with those of Lackie (1983) and may represent a loophole in the immune surveillance to allow *Moniliformis* to survive.

4. Capsules *in vivo* and *in vitro* contained the enzymes, acid phosphatase and peroxidase, and phagocytic cells are also incorporated into the capsules.

5. Coating the substratum with BSA reduced the cell spreading *in vitro* compared with glass but this effect was not dose dependent.

6. Infection with *Hymenolepis* or Zs pre-treatment did not affect haemocyte spreading indicating that the thinner capsules after

Hymenolepis infection was due to a decrease in recruitment rather than an enhanced ability of cells to spread.

CHAPTER 5 - ADHESION IN THE FLOW CHAMBER

CHAPTER 5 - ADHESION IN THE FLOW CHAMBER

INTRODUCTION

Adhesion - The Flow Chamber

Insects have an open circulatory system in which flow is maintained by a dorsal heart. The blood occupies the general body cavity and bathes the internal organs. Haemocytes present in the blood are in direct contact with the epithelial lining of the haemocoel and may adhere during defence reactions. This process is known as margination and is probably responsible for variations in the circulating cell number.

In vivo observations of margination in invertebrates are rare. Haemolymph flow through wing veins was observed by Arnold (1959). The haemocytes were seen to jostle through the narrow vessels without sticking to one another. Such observations are interesting but the flow conditions and environment cannot be experimentally manipulated, therefore, details of adhesion and margination require in vitro investigation.

Several assays are used to assess adhesion by blood cells in vitro; these include the aggregation or nodule assay (see Chapter 3) and capsule formation in vivo and in vitro (see Chapter 4). The nodule assay measures cell-cell adhesion whereas, capsule formation consists of an initial cell-substratum interaction followed by cell-cell adhesion in the later stages. Cell-cell interactions clearly are relevant in the immune response, but interpretation of the cellular changes which induce adhesion is difficult. Cell-substratum adhesion, especially under conditions of flow, models the adhesion to haemocoel linings and permits more detailed investigation of the molecular basis

of adhesion eg. receptor-ligand binding.

An in vitro model for cell adhesion in the presence of flow was used by Forrester and Lackie (1984) to investigate neutrophil adhesion. This system avoids the problem of standard static assays for adhesion, in which spreading may occur before adhesion because of the effects of gravity. In static assays, round cells settling out of suspension become flattened which increases the cell-substratum contact area so decreasing the likelihood of subsequent detachment. Cells exposed to the flow conditions of the chamber must adhere before spreading. Furthermore, cells marginating in vivo continue to be exposed to the shear forces associated with fluid movement. The flow chamber permits monitoring of the kinetics of adhesion since the instantaneous associations and disassociations of cells exposed to such distraction forces can be followed. An added advantage is the ease with which the substratum can be manipulated to investigate the effect of different molecules on adhesion.

In this chapter, the flow chamber is used to model haemocyte adhesion in the haemocoel and to investigate the effect of in vivo immune manipulation on cell adhesion.

Infection and Adhesion

Before haemocytes can fulfill their primary role as immune effectors, they must recognise and adhere to the invader. Several defence responses of invertebrates, nodule formation, encapsulation and margination for example, require alterations in the adhesiveness of the blood cells. From the work presented in previous chapters, it is clear that cell adhesion is influenced by soluble molecules (β1,3-glucans) and infection with Hymenolepis and Moniliformis.

This raises several interesting questions on the cellular adhesion and recognition of foreignness by haemocytes. How do the cells recognise the invader? What is the stimulus for changing from non-adherent circulating cells to adherent cells which then participate in the immune response? However, the main question addressed in this chapter is; do haemocytes from stimulated immune systems express different surface molecules compared with cells from naive animals?

Recognition and Adhesion

Until recently, studies on the lectins, also known as agglutinins, of invertebrates reported their chemical structure and molecular specificity only with little direct investigation of their immunological role (Vasta, et al., 1982a; Vasta et al., 1982b; Vasta and Cohen, 1984a; 1984b). Lectins are proteins or glycoproteins which bind specifically to sugar residues and are important in phagocytosis (for review see Sharon, 1984) and agglutination.

Although several authors have surmised about the role of lectins in invertebrate immune surveillance and protection (for reviews see Cooper and Lemmi, 1981; Yeaton, 1981; Vasta and Marchalonis, 1985) studies have concentrated on the opsonic (Renwrantz and Stahmer, 1983; Ratcliffe and Rowley, 1983; Bradley et al., 1989; Fryer and Bayne, 1989; Drif and Brehelin, 1989) and agglutinating (Lackie, 1981a; Vasta et al., 1982b; Ingram and Molyneux, 1990) properties of the serum molecules and relatively little is known of the importance of cell membrane lectins in the defence response.

Because of their ability to agglutinate cells, lectins are prime candidates for recognition molecules (Lackie, 1981a; Vasta and

Marchalonis, 1985) in animals which lack immunoglobulin. Recognition of non-self and cell-cell recognition and co-operation may be explained by a lectin-ligand interaction at the cell surface. Furthermore, cell membrane lectins could serve as receptors to bind directly with carbohydrate molecules on the surface of invaders to initiate an immune response. Thus, haemocyte binding to foreign particles and recognition of "self" versus "non-self" could be mediated via a receptor-ligand mechanism.

The haemocytes of the cockroach, P. americana have a galactose-specific lectin of 35Kd present in granules and exposed on the plasma membrane (Lackie and Vasta, 1986) and a huge haemolymph lectin (1500Kd) consisting of many 30Kd subunits (Kubo and Natori, 1987). Recent studies in molluscs on the carbohydrate specificities of lectins have implied that haemolymph component is not simply a soluble form of the membrane-bound molecule (Fryer et al., 1989).

Lackie and Vasta (1988) tested the hypothesis that the lectins of invertebrates may be involved in immunorecognition. These authors used assays for haemocyte behaviour to investigate the cellular response of the cockroach, P. americana, to galactose-rich (porcine stomach mucin) and sialic acid-rich glycoproteins (bovine submaxillary mucins) (see Gottschalk, 1966 for general information on glycoproteins). The former conjugated to Sepharose beads stimulated thicker capsules than sialic acid-rich glycoproteins, providing evidence for carbohydrate specificity in the immune response. The experiments described here were carried out to investigate whether this lectin is involved in the initial stages of non-self recognition by mediating cell adhesion. By measuring the adhesion of haemocytes to the glycoproteins, information may be obtained on the nature of adherent cell subpopulations involved

in immune reactions eg. encapsulation and nodule formation.

The ability of more cells to stick to a galactose-rich molecule compared with a sialic acid-rich molecule implies a degree of specificity in cell behaviour which might reflect different haemocyte populations. Using this assay, information on the specific adhesivity of cell subpopulations in vitro can be obtained which could help in the identification of the functional characteristics of the plasmatocyte subpopulations.

Comparison of adhesion by haemocytes from naive or immune manipulated animals may improve the understanding of the changes in cell surface lectins or cell populations which accompany immune reactivity. Enhanced expression of the cell membrane lectin after infection may indicate immune recognition of an invader and improved immune surveillance.

For a parasite to survive while in contact with the immune defences of the host, it may either evade or suppress host immunity. In a suppressed immune system, either the recognition arm or the effector arm of the defence system may be affected. The experiments presented below attempt to clarify how the immune system recognises an invader and responds to the infection. Evidence for either suppression or evasion of immune surveillance by Moniliformis might be derived from these studies.

RESULTS

This chapter will deal with the effect of immune manipulation on adhesion of haemocytes in the flow chamber. Since the haemocytes of P. americana carry a galactose-specific lectin, adhesion to galactose-rich (PSM) and sialic-acid rich (BSM) glycoproteins has been studied. It was expected that haemocytes would adhere more readily to the PSM via a specific receptor-ligand interaction. Furthermore, since lectins have been proposed as being involved in the recognition of foreignness, cell adhesion after immune manipulation with soluble molecules (Zymosan supernatant) or parasites (Hymenolepis and Moniliformis has been investigated.

5.1. Behaviour of Haemocytes in the Flow Chamber

The haemocytes of P. americana showed differential adhesiveness under flow conditions. Haemocytes tended to roll along the substratum, adhere briefly then detach as the shear force exerted from the fluid broke the adhesions. At higher flow rates cells did not form stable adhesions. Cells which formed longer-lasting adhesions tended to spread rapidly (5-10mins) and, once spread, did not detach. As judged by their ability to spread, it is most likely that these adherent cells were viable plasmatocytes. Round, phase-bright cells also adhered in the flow chamber and these were probably coagulocytes. Haemocytes were occasionally captured in coagulum from cells which had adhered then lysed. In these instances, streamers of cells were collected downstream. In addition, cells often accumulated from the fluid flow around adherent cells so that aggregations similar to nodules were formed.

5.2. Effect of Zymosan Injection on Adhesion

The results are shown in Tables/Figs. 5.2.1 and 5.2.2.

Zs decreases the circulating cell number and stimulates nodule formation *in vivo* (Gunnarsson and Lackie, 1985; Chapter 3) which suggests a change in cell adhesiveness in response to this soluble molecule. In these experiments adhesion to the glycoproteins (PSM and BSM) has been investigated to find whether specific cell adhesion is altered after Zs pre-treatment.

Tables 5.2.2. and 5.2.1 show clearly that haemocytes from both control and experimental animals adhere more readily to PSM than BSM and these differences are significant. Zs had little effect on cell adhesion after 1hr (Fig. 5.2.1). However, three hours after pre-treatment with Zs the haemocytes became less adherent to the PSM and more adherent to the BSM compared with the naive and saline-injected controls ($p<0.05$ in all cases, Mann Whitney two sample test). This trend was observed regardless of which glycoprotein the cells encountered first. This shows that the effect is real and not due simply to the order of the glycoproteins in the flow chamber. Saline injections caused a similar but less marked effect than Zs pre-treatment.

These results suggest that immune stimulation alters the specific adhesivity of the plasmatocyte population, perhaps by modifying the lectin binding characteristics of the cells. This may reflect enrichment or depletion of cell subpopulations as the cells participate in the *in vivo* response, eg. nodule formation, to the wound or the β1,3-glucan molecules.

5.3. Effect of Infection with *H. diminuta* on Adhesion

Hymenolepis oncospheres are readily recognised by the cellular immune system of the cockroach and subsequently become encapsulated by the blood cells. The purpose of the following experiments was to find if adhesion by the blood cells to the glycoproteins may be affected by the parasites. This may provide some insight into the mechanism of cellular recognition of non-self in the insect. Figures 5.3.1. and 5.3.2. show adhesion of haemocytes to BSM and PSM after infection with *Hymenolepis*.

One day after feeding *Hymenolepis* to cockroaches haemocyte adhesion to BSM is significantly higher ($p<0.05$, Mann Whitney two sample rank test) compared with adhesion by haemocytes from naive controls (Fig. 5.3.1.) and this effect is independent of which glycoprotein the cells contact first. *Hymenolepis* does not affect adhesion to PSM 1 day after infection. In control animals there are significantly more cells adherent to PSM compared with BSM (Table 5.3.1.). After infection with *Hymenolepis*, the proportion of cells sticking to the BSM becomes comparable with the proportion adhering to the PSM.

Three days after feeding with *Hymenolepis*, a larger proportion ($p<0.05$) of cells adhere to BSM, compared with the naive and saline-injected controls (Fig. 5.3.2.). *Hymenolepis* also significantly ($p<0.05$) enhances the adhesion of haemocytes to PSM (Fig. 5.3.2.a).

These results show that *Hymenolepis* does alter the adhesive properties and, perhaps, the discriminatory abilities of the circulating haemocytes during the course of the infection.

5.4. Effect of Infection with *M. moniliformis* on Adhesion

Moniliformis acanthors are encapsulated during the early stages of

haemocoelic development but by stage II/III acanthellae, the capsules are no longer evident. Haemocyte adhesion to defined substrata (BSM and PSM) has been studied to compare the effect of early and late stages of Moniliformis infection on cell adhesion to defined substrata. Changes in the discriminatory abilities of the haemocytes in the later stages of Moniliformis infections may provide information on the immune recognition and the survival mechanism of the parasite.

Figures 5.4.2.a-b show that infection with acanthors of Moniliformis does not significantly alter the adhesion of haemocytes to the glycoproteins compared with controls (all values non-significant using Mann Whitney two sample test), although there is a slight increase in the proportion of cells which adhere to the PSM.

Enhanced adhesion to glycoprotein-coated glass by haemocytes from parasite-infected (Hymenolepis) animals is an interesting observation since recognition of non-self may be partially dependent on the galactose lectin-ligand interaction. To test this idea, adhesion by haemocytes from Moniliformis acanthellae-infected animals was studied.

All experimental treatments, including Moniliformis acanthellae infections, reduced cell adhesion to PSM compared with naive controls and this was evident whether the cells encountered BSM or PSM first. These results suggest that experimental handling alters cell adhesiveness. However, these differences were not significant when compared with the Mann Whitney two sample rank test and are due to large experimental variation between the naive controls. In these experiments adhesion to PSM is much higher than in previous and later experiments (see Figs. 5.2.2., 5.3.1., 5.3.2.). Infections with Moniliformis acanthellae, in contrast with Hymenolepis infections, had no effect on the adhesion of cells to BSM.

The data can be summarised quite simply: there is no detectable alteration in haemocytic adhesion as a consequence of infection with Moniliformis.

5.5. Adhesion to Asialo Glycoproteins

Since native PSM contains only approximately 1% bound sialic acids, desialylation will alter the molecule very little. However, removing the sialic acid from the BSM molecule is likely to have a more profound effect on the charge and structure of the molecule.

Table 5.5.2. shows that desialylating PSM changes the properties of the molecule sufficiently so that significantly fewer ($p<0.01$) haemocytes can adhere compared with the native PSM. This is more pronounced when the cells encounter the native molecule first, although a similar, but not significant, effect is seen if the cells contact the asialo and then the native PSM.

Desialylation of BSM has some effect on the adhesion of haemocytes in the flow chamber. Table 5.5.1. shows that significantly more ($p<0.05$) cells from naive animals adhere to the asialo-BSM, but only when the cells are presented with the desialylated BSM before the native BSM. The results represented in Figure 5.5.1.a show that after infection with Hymenolepis, significantly fewer ($p<0.05$) cells adhere to the asialo-BSM. This is most likely due to cells adhering to the native BSM before they reach the asialo-BSM since there is no difference in adhesion to asialo-BSM between control and experimental infections in Figure 5.5.1.b (asialo-BSM to native BSM). Although adhesion to the BSA-coated strip located between the two glycoproteins in Figure 5.5.1.b seems much higher than expected, this value is not

significantly different when the control and experimental animals are compared. This seems to have arisen because of large experimental variations in the cell samples from animals fed immature Hymenolepis.

ADHESION 1 HR AFTER ZYMOSAN PRE-TREATMENT

Table 5.2.1

Results are expressed as a proportion of the initial BSA-coated glass strip which was normalised to 100% (mean % of cells adhering to BSA +s.d.). Data are presented from 6 experiments.
a=p<0.01 and b=p<0.05 compared to BSM, using Mann Whitney two sample rank test.

Treatment	Glass Coating				
	BSA	PSM	BSA	BSM	BSA
Naive	100	96.0+28.7[a]	92.3+16	21.6+9.3	109.1+43.9
Saline	100	105.6+35.9[a]	99.4+38.3	16.9+8.8	133.4+69.5
Zymosan	100	121.8+78.2[b]	105.0+46.5	14.1+3.3	155.5+105.0

Treatment	BSA	BSM	BSA	PSM	BSA
Naive	100	17.0+8.0	83.4+23.9	94.6+17.9[a]	100.8+17.8
Saline	100	18.5+9.7	90.8+29.0	97.3+21.6[a]	96.2+18.1
Zymosan	100	14.9+3.3	107.1+25.8	84.2+27.9[a]	89.3+26.5

HAEMOCYTE ADHESION 3 HR AFTER ZYMOSAN PRE-TREATMENT

Table 5.2.2

Results are expressed as a proportion of the initial BSA-coated glass strip which was normalised to 100% (mean % of cells adhering to BSA +s.d.). Data are presented from 6 experiments.
a=p<0.01 and b=p<0.05 compared to BSM, using Mann Whitney two sample rank test

Treatment	Glass Coating				
	BSA	PSM	BSA	BSM	BSA
Naive	100	124.9+24.2[a]	145.0+66.5	16.0+14.1	111.2+36.5
Saline	100	101.1+32.4[a]	81.0+34.3	16.4+14.4	93.4+31.1
Zymosan	100	82.3+17.3[a]	98.8+14.9	41.2+17.6	87.5+17.6

Treatment	BSA	BSM	BSA	PSM	BSA
Naive	100	16.9+7.2	122.6+33.7	126.9+36.5[a]	123.4+58.6
Saline	100	29.1+37.0	104.8+25.9	119.1+61.2[b]	78.1+16.6
Zymosan	100	40.5+13.8	103.5+38.8	91.5+19.4[a]	107.6+35.0

FIGURE 5.2.1.a.= PSM TO BSM

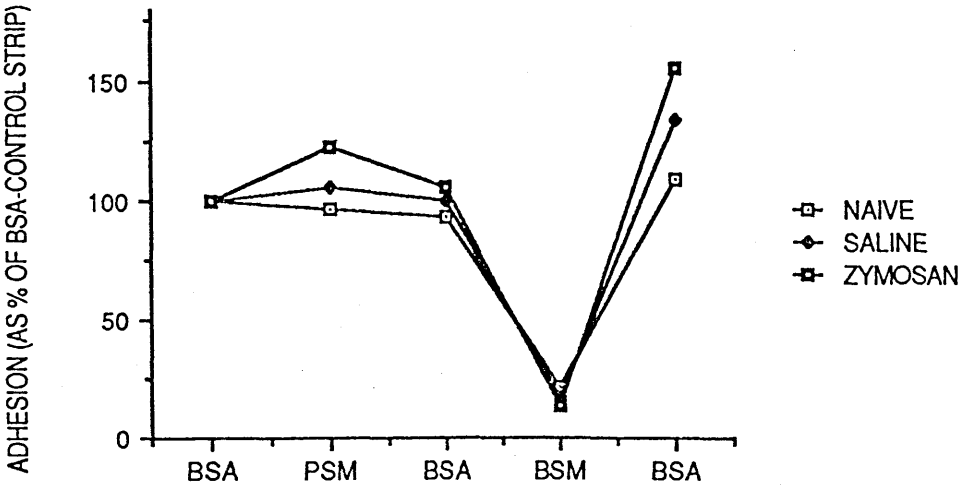


FIGURE 5.2.1.b.= BSM TO PSM

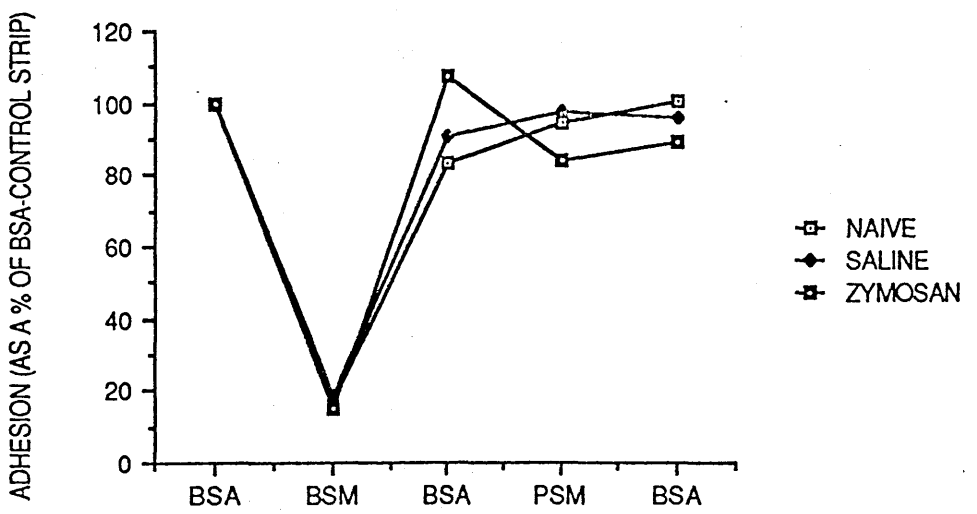


Figure 5.2.1.
Adhesion to porcine stomach mucin (PSM) and bovine submaxillary mucin (BSM) under conditions of flow, by haemocytes from cockroaches injected with Zs 1hr previously. Results are expressed as a mean % of cells adhering to the initial bovine serum albumin (BSA)- coated glass strip. Number of experiments = 6.

FIGURE 5.2.2.a. = PSM TO BSM

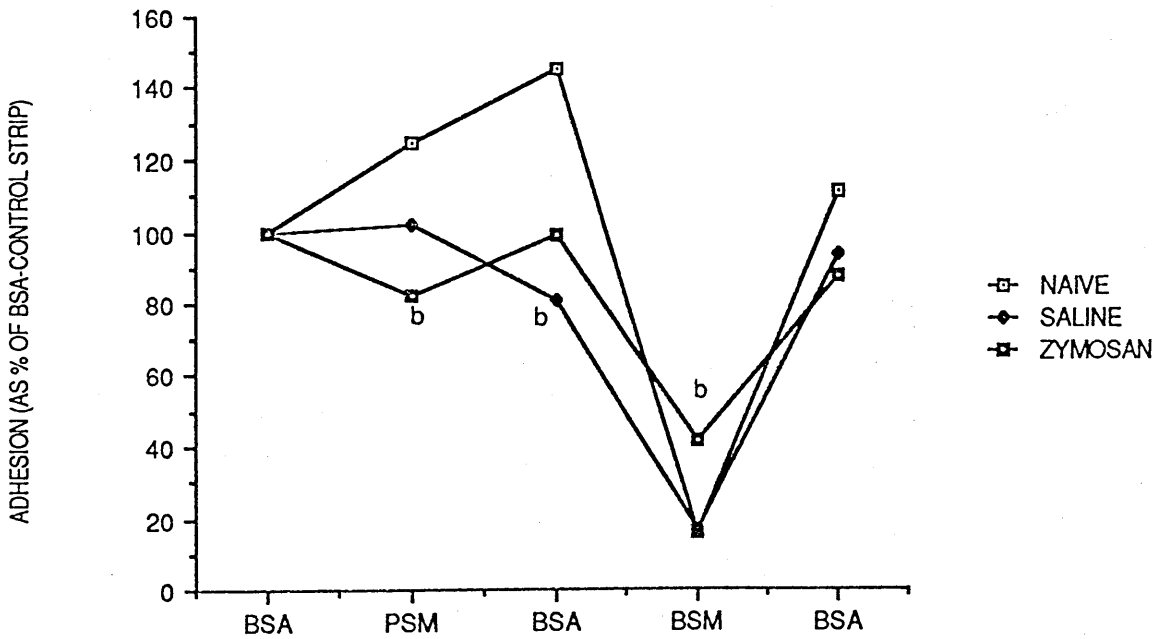


FIGURE 5.2.2.b. = BSM TO PSM

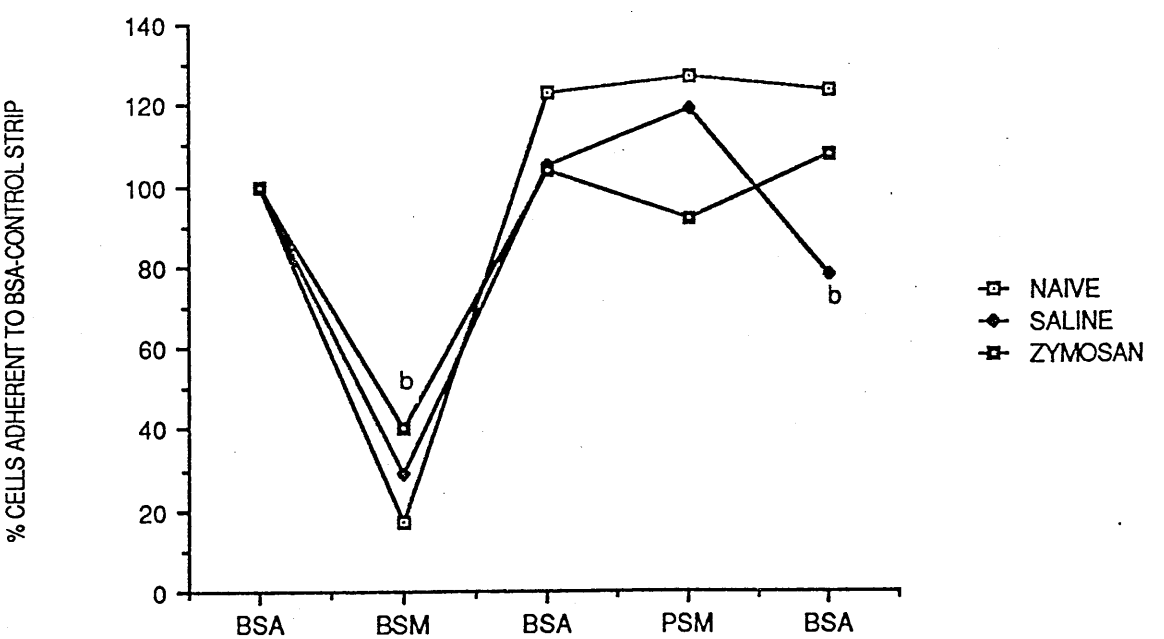


Figure 5.2.2.
Adhesion to porcine stomach mucin (PSM) and bovine submaxillary mucin (BSM) under conditions of flow, by haemocytes from cockroaches injected with Zs 3hr previously. Results are expressed as a mean % of cells adhering to the initial bovine serum albumin (BSA)- coated glass strip. Number of experiments = 6.
b<0.05 compared to naive control using Mann Whitney two sample rank test.

CELL ADHESION 1 DAY AFTER HYMENOLEPIS INFECTION

Figure 5.3.1

Results are expressed as a proportion of the initial BSA-coated glass strip which was normalised to 100% (mean % of cells adhering to BSA +s.d.). Data are presented from 4 experiments.
b=p<0.05 compared with BSM using Mann Whitney two sample rank test.
Hd = Hymenolepis, Imm. = immature, Mat. = mature

Treatment	Glass Coating				
	BSA	PSM	BSA	BSM	BSA
Naive	100	109.5+39.0[b]	81.7+9.3	41.7+8.2	74.3+33.1
Imm. Hd	100	97.8+21.0[b]	94.7+14.6	30.1+7.4	97.1+31.0
Mat. Hd	100	113.2+21.0	93.0+15.4	81.8+48.0	105+62.9

Treatment	BSA	BSM	BSA	PSM	BSA
Naive	100	48.1+7.7	127.1+36.6	111.2+33.4[b]	86.2+9.3
Imm. Hd	100	34.0+20.2	94.4+20.5	90.0+20.1[b]	68.0+20.6
Mat. Hd	100	70.6+11.0	111.6+41.1	92.7+21.0	95.0+8.5

ADHESION 3 DAYS AFTER HYMENOLEPIS INFECTION

Table 5.3.2

Results are expressed as a proportion of the initial BSA-coated glass strip which was normalised to 100% (mean % of cells adhering to BSA +s.d.). Data are presented from 6 (naive) or 3 (parasite) experiments.
a=p<0.01 compared with BSM using Mann Whitney two sample rank test.
Hd = Hymenolepis, Imm. = immature, Mat. = mature

Treatment	Glass Coating				
	BSA	PSM	BSA	BSM	BSA
Naive	100	97.6+8.7[a]	116.9+35.0	22.5+19.4	128.7+41.4
Imm. Hd	100	112.4+17.8	67.1+13.7	32.4+12.3	63.6+40.3
Mat. Hd	100	185.1+23.4	99.9+20.6	83.3+11.4	110.7+20.4

Treatment	BSA	BSM	BSA	PSM	BSA
Naive	100	12.4+8.8	111.4+28.8	112.4+38.5[a]	106.8+29.0
Imm. Hd	100	61.3+7.7	106.1+8.9	113.1+12.6	85.7+9.4
Mat. Hd	100	113.9+59.6	130.6+52.4	142.9+69.2	147.3+115.4

FIGURE 5.3.1.a. = PSM TO BSM

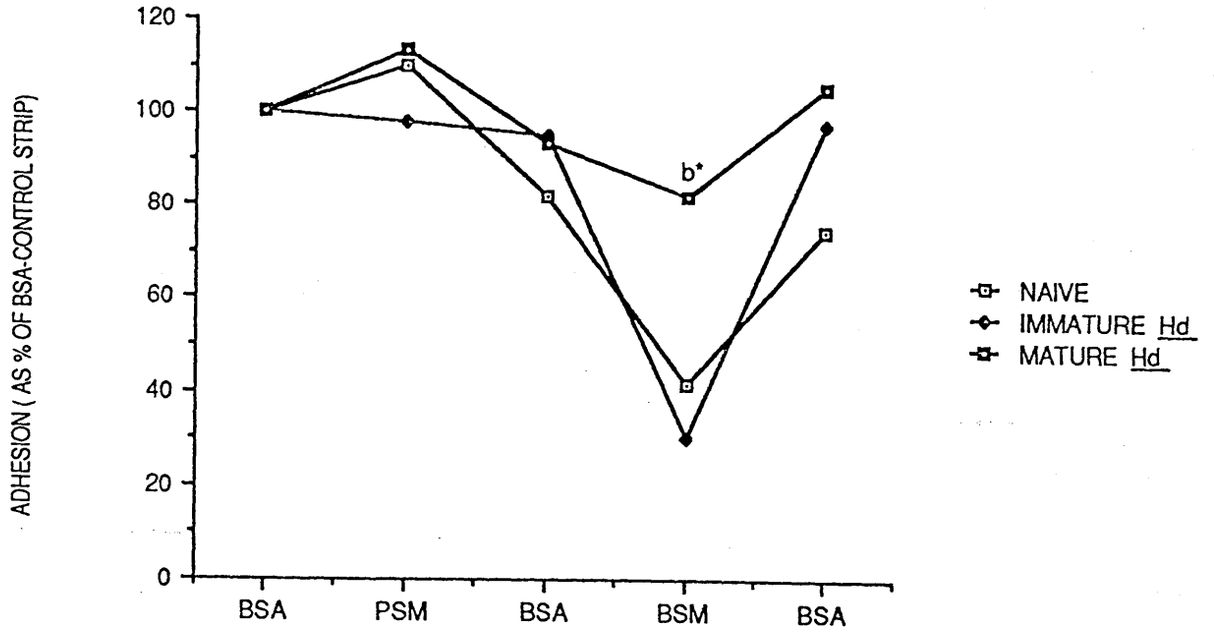


FIGURE 5.3.1.b. = BSM TO PSM

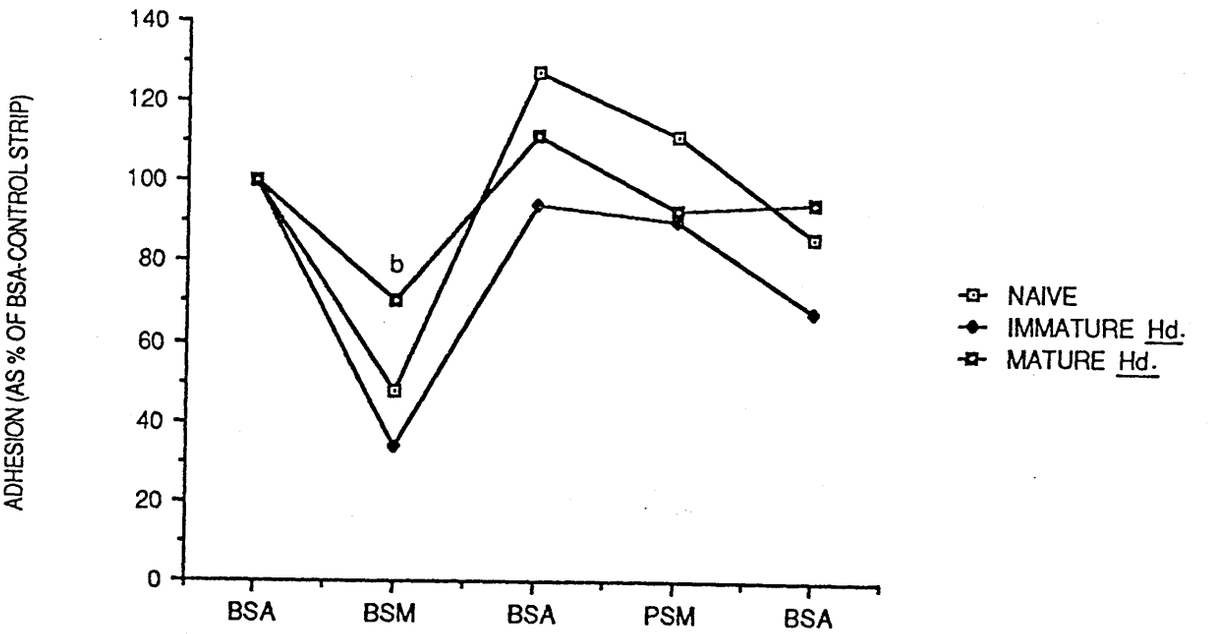


Figure 5.3.1.
Adhesion to porcine stomach mucin (PSM) and bovine submaxillary mucin (BSM) under conditions of flow, by haemocytes from cockroaches fed Hymenolepis diminuta 1 day previously. Results are expressed as a mean % of cells adhering to the initial bovine serum albumin (BSA)-coated glass strip. Number of experiments = 4.
b=$p<0.05$ compared with naive control using Mann Whitney two sample rank test.
b*=$p<0.05$ compared with imm. control using Mann Whitney two sample rank test.

Hd. = Hymenolepis diminuta

FIGURE 5.3.2.a. = PSM TO BSM

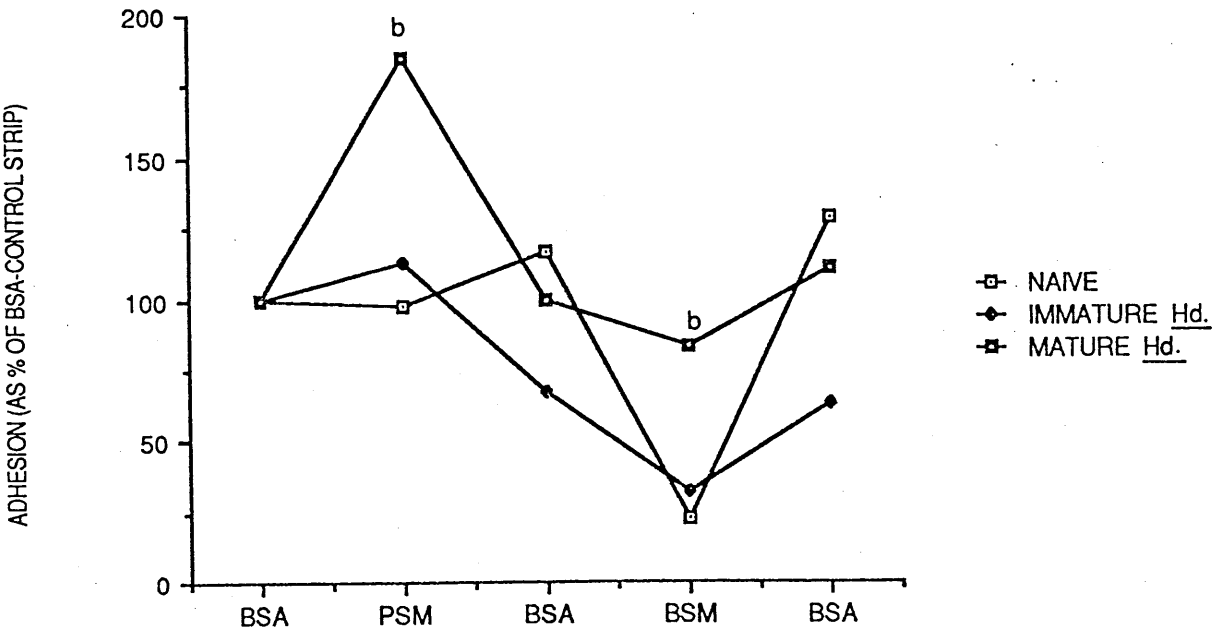


FIGURE 5.3.2.b. = BSM TO PSM

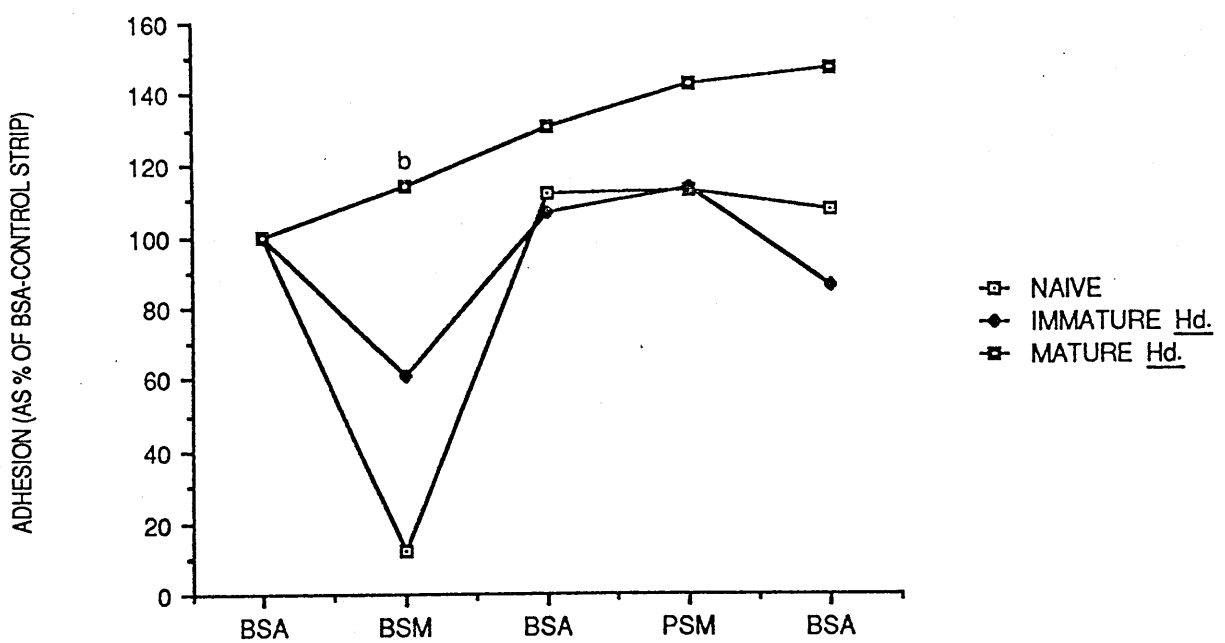


Figure 5.3.2.
Adhesion to porcine stomach mucin (PSM) and bovine submaxillary mucin (BSM) under conditions of flow, by haemocytes from cockroaches fed Hymenolepis diminuta 3 days previously. Results are expressed as a mean % of cells adhering to the initial bovine serum albumin (BSA)-coated glass strip. Number of experiments = 3.
b=$p<0.05$ compared with naive control using Mann Whitney two sample rank test.

Hd. = Hymenolepis diminuta

CELL ADHESION IN MONILIFORMIS (ACANTHELLAE)-INFECTED COCKROACHES

Table 5.4.1

Results are expressed as a proportion of the initial BSA-coated glass strip which was normalised to 100% (mean % of cells adhering to BSA +s.d.). Data are presented from 3 experiments.

No significant differences between adhesion to BSM compared with PSM in each treatment.

Treatment	Glass Coating				
	BSA	PSM	BSA	BSM	BSA
Naive	100	190.3+38.5	92.4+33.4	47.9+21.5	121.9+52.7
Medium	100	105.0+19.4	67.7+11.5	12.4+6.2	73.3+13.3
Ovariole	100	109.4+13.6	86.7+2.1	28.9+12.8	103.1+7.6
Acanthellae	100	112.4+10.3	66.0+38.6	33.2+5.7	98.2+34.4
Treatment	BSA	BSM	BSA	PSM	BSA
Naive	100	37.5+23.3	112.2+26.7	205.2+153.3	106.8+17.6
Medium	100	38.8+12.2	112.5+8.0	145.3+37.4	123.4+7.3
Ovariole	100	29.5+5.3	96.7+15.1	120.0+20.7	90.2+12.8
Acanthellae	100	15.1+12.4	134.9+34.9	136.8+103.6	96.2+28.3

ADHESION IN MONILIFORMIS (ACANTHOR)-INFECTED COCKROACHES

Table 5.4.2

Results are expressed as a proportion of the initial BSA-coated glass strip which was normalised to 100% (mean % of cells adhering to BSA +s.d.). Data are presented from 4 experiments.

b=$p<0.05$ compared with BSM using Mann Whitney two sample rank test.

Mm = Moniliformis

Treatment	Glass Coating				
	BSA	PSM	BSA	BSM	BSA
Naive	100	121.5+34.8[b]	67.0+17.3	37.4+11.9	55.2+9.1
H.I. Mm	100	137.8+61.8[b]	60.4+22.9	32.5+18.3	80.4+18.9
Live Mm	100	152.7+98.7[b]	79.1+41.2	18.1+7.1	66.6+34.3
Treatment	BSA	BSM	BSA	PSM	BSA
Naive	100	54.7+13.0	92.0+23.0	80.8+35.2	75.4+14.4
H.I. Mm	100	69.5+31.5	92.0+23.0	146.3+68.1	61.3+5.4
Live Mm	100	65.0+25.3	123.7+43.1	133.4+34.8	74.1+28.3

FIGURE 5.4.1.a. = PSM TO BSM

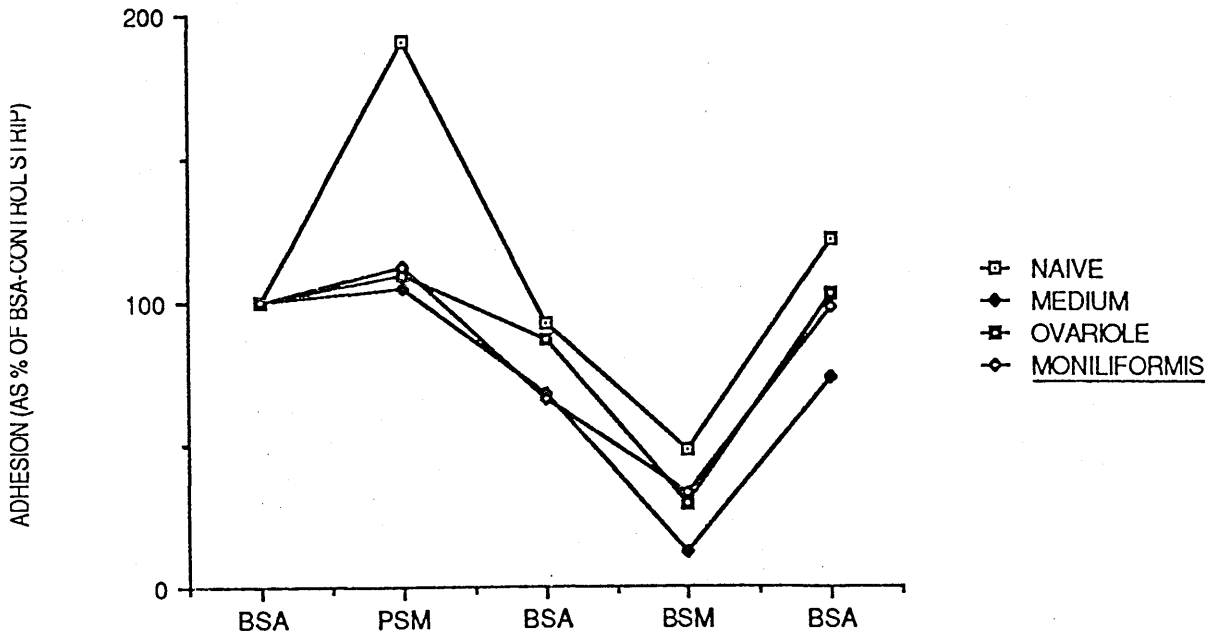


FIGURE 5.4.1.b. = BSM TO PSM

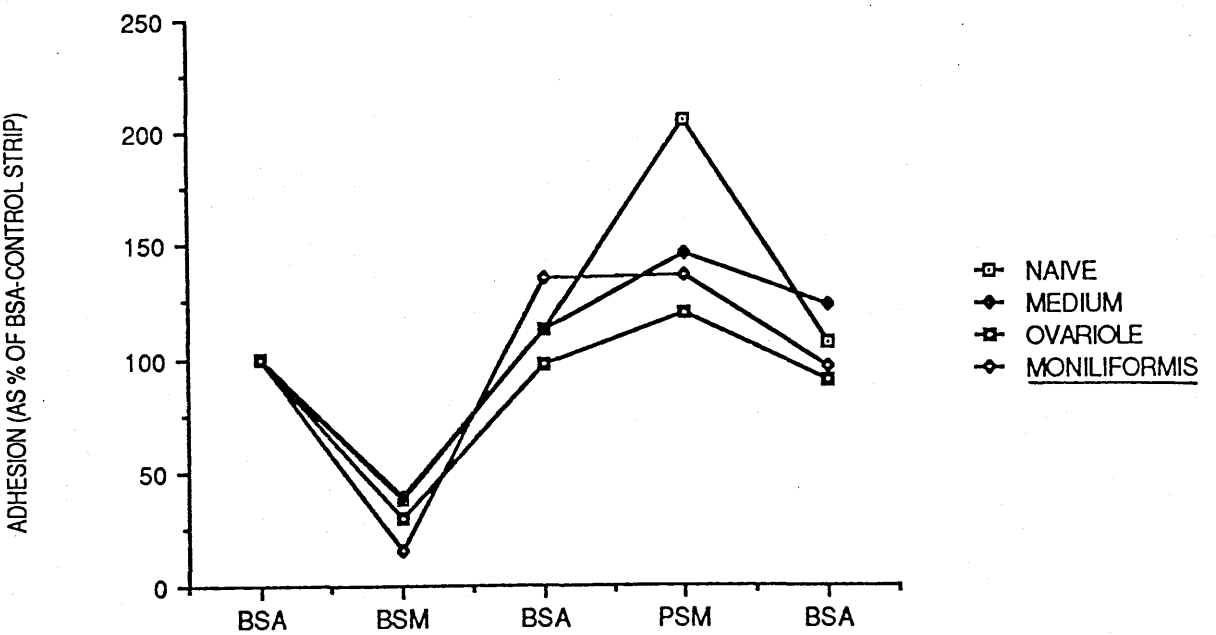


Figure 5.4.1.
Adhesion to porcine stomach mucin (PSM) and bovine submaxillary mucin (BSM) under conditions of flow, by haemocytes from cockroaches infected with acanthellae of Moniliformis moniliformis. Results are expressed as a mean % of cells adhering to the initial bovine serum albumin (BSA)- coated glass strip. Number of experiments = 3. All values non-significant using Mann Whitney two sample rank test.

FIGURE 5.4.2.a. = PSM TO BSM

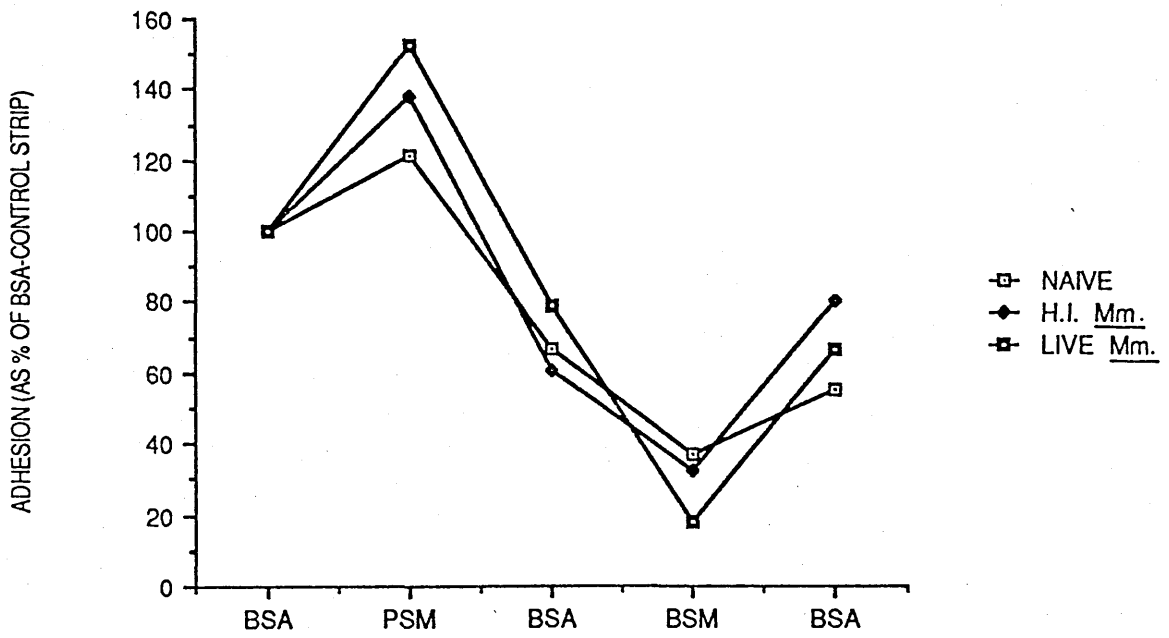


FIGURE 5.4.2.b. = BSM TO PSM

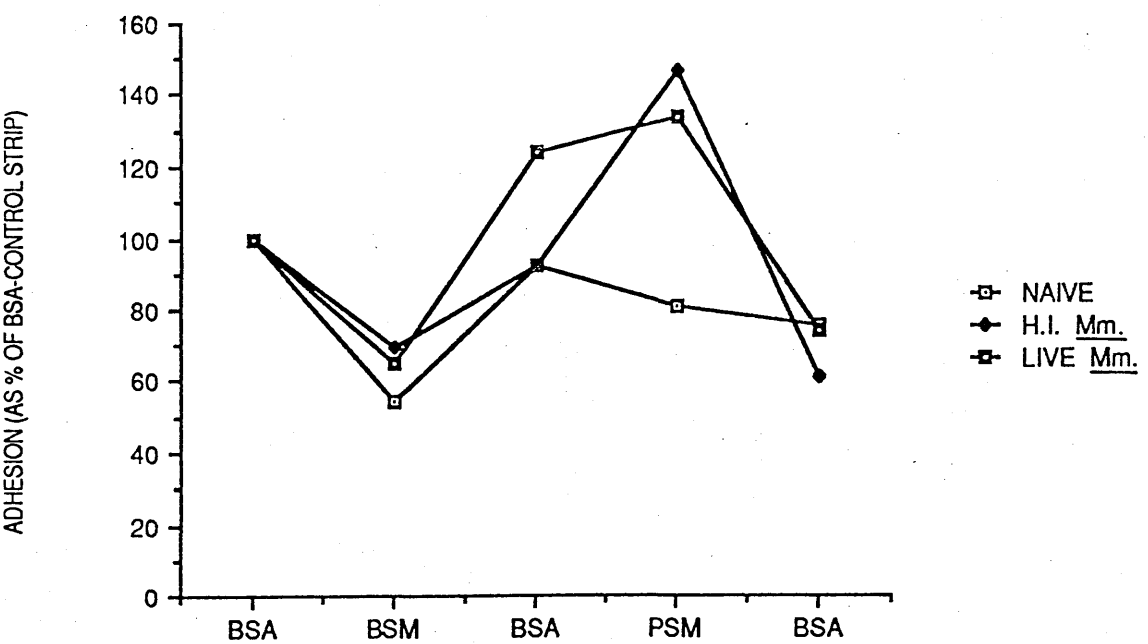


Figure 5.4.2.
Adhesion to porcine stomach mucin (PSM) and bovine submaxillary mucin (BSM) under conditions of flow, by haemocytes from cockroaches fed with acanthors of Moniliformis moniliformis 2 days previously. Results are expressed as a mean % of cells adhering to the initial bovine serum albumin (BSA)- coated glass strip. Number of experiments=3. All values non-significant using Mann Whitney two sample rank test.

Mm = Moniliformis moniliformis

ADHESION TO ASIALO-BSM 3 DAYS AFTER *HYMENOLEPIS*-INFECTION

Table 5.5.1

Results are expressed as a proportion of the initial BSA-coated glass strip which was normalised to 100% (mean % of cells adhering to BSA +s.d.). Data are presented from 6(naive) or 4(parasite) experiments. aBSM=asialo-bovine submaxillary mucin, nBSM=native bovine submaxillary mucin, *Hd* = *Hymenolepis*, Imm. = immature, Mat. = mature
b=$p<0.05$ compared to asialo-BSM using Mann Whitney two sample rank test.

Treatment	Glass Coating				
	BSA	aBSM	BSA	nBSM	BSA
Naive	100	49.6+12.4	79.3+16.2	21.1+13.1[b]	71.6+21.2
Imm. *Hd*	100	49.4+31.9	141.9+59.7	31.3+13.5	102.8+37.5
Mat. *Hd*	100	47.5+27.3	65.5+27.7	49.8+19.6	56.4+19.3

Treatment	BSA	nBSM	BSA	aBSM	BSA
Naive	100	40.8+5.4	102.5+16.9	53.8+11.8	91.4+32.7
Imm. *Hd*	100	48.2+20.1	124.4+44.1	50.0+11.7	91.9+37.1
Mat. *Hd*	100	53.4+37.6	95.8+22.9	28.6+15.6	65.5+28.3

ADHESION TO ASIALO-PSM BY HAEMOCYTES FROM NAIVE COCKROACHES

Figure 5.5.2

Results are expressed as a proportion of the initial BSA-coated glass strip which was normalised to 100% (mean % of cells adhering to BSA +s.d.). Data are presented from 6 experiments.
aPSM=asialo-porcine stomach mucin, nBSM=native porcine stomach mucin.
a=$p<0.01$ compared to asialo-PSM using Mann Whitney two sample rank test.

Coating	BSA	nPSM	BSA	aPSM	BSA
	100	108.0+9.0[a]	81.0+18.3	52.9+15.9	84.4+23.8

Coating	BSA	aPSM	BSA	nPSM	BSA
	100	65.8+23.3	93.5+12.8	118.7+42.2	67.1+17.3

FIGURE 5.5.1.a. = NATIVE-BSM TO ASIALO-BSM

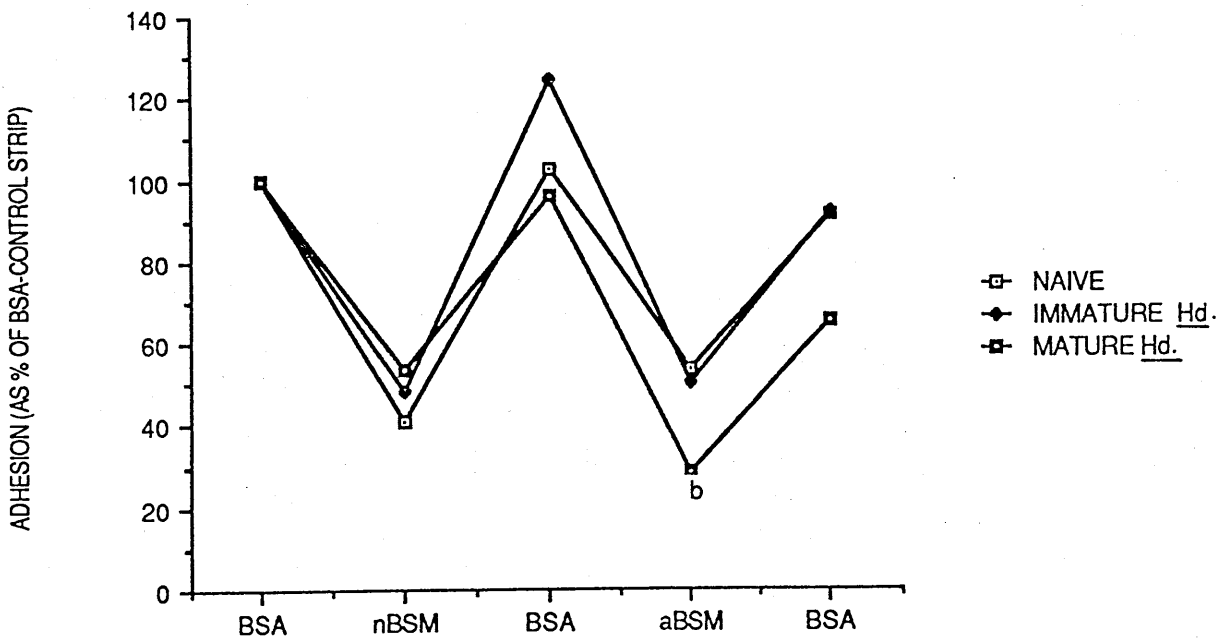


FIGURE 5.5.1.b. = ASIALO-BSM TO NATIVE-BSM

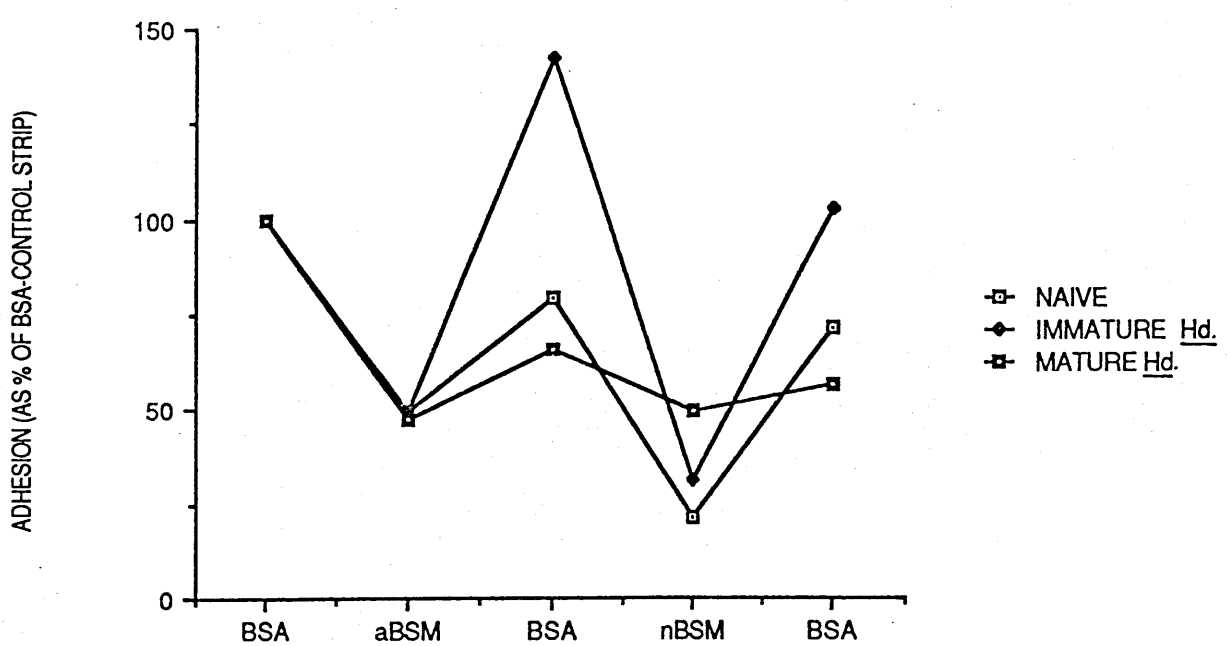


Figure 5.5.1.
Adhesion to native and asialo-bovine submaxillary mucin (nBSM and aBSM) under conditions of flow, by haemocytes from cockroaches fed Hymenolepis diminuta 3 days previously. Results are expressed as a mean % of cells adhering to the initial bovine serum albumin (BSA)-coated glass strip. Number of experiments = 6 (naive) or 4 (parasite).
b<0.05 compared with naive control using Mann Whitney two sample rank test.

Hd. = Hymenolepis diminuta

DISCUSSION

The Flow Chamber

Alterations in the stickiness of cells or of the substratum is necessary for several immune responses eg. nodulation and encapsulation. The adhesiveness of invertebrate blood cells has received a considerable amount of attention and can be assayed indirectly and quantitatively by measuring capsule thickness (Lackie, 1983) or nodule numbers (Gunnarsson and Lackie, 1985). Although detecting gross changes in adhesion, these assays do little to clarify the molecular basis of non-self discrimination and adhesive interactions.

In the present study, an *in vitro* assay used for investigating adhesion by vertebrate blood cells (Forrester and Lackie, 1984) has been modified for use with insect haemocytes. Using this technique, it has been possible to study haemocytic adhesion at the molecular level. Since the haemocytes retained the ability to distinguish surface properties under carefully controlled conditions, the flow chamber will have multiple applications in investigating the cellular immune response of invertebrates.

The Galactose-specific Cockroach Lectin

The haemocytes (Lackie and Vasta, 1986) and haemolymph (Kubo and Natori, 1987) of *P. americana* contain a galactose-specific lectin which may mediate some aspects of the immune response. Sepharose beads conjugated to either galactose-rich or desialylated glycoproteins are more thickly encapsulated by cockroach haemocytes than similar beads coated with sialic acid-rich glycoproteins (Lackie and Vasta, 1986).

In the present study, the importance of the cellular galactose-specific lectin in cell adhesion in flow conditions has been investigated. Haemocytes derived from naive animals adhered well under flow conditions to the galactose-rich glycoprotein (PSM), less well to the protein (BSA) and poorly to the sialic acid-rich glycoprotein (BSM). These results are in agreement with the findings of Lackie and Vasta (1988) for the nodule inducing activity of these molecules, and provide further evidence for a role of the membrane-bound lectin in cell behaviour.

Bovine submaxillary mucin (BSM), which contains little galactose (1.4% (Horowitz and Pigman, 1977)), was used for comparison with cell adhesion to the galactose-rich molecule, porcine stomach mucin (PSM). Haemocytes which express a galactose-specific lectin on the cell surface might be expected to stick to PSM but not BSM. The results suggest that the cellular adhesion via this receptor-ligand mechanism is quite specific because fewer cells (see Table 5.2.2.) adhered to the BSM compared with the PSM.

However, BSM was not completely non-adherent since some cells could stick to the BSM-coated glass and this can be interpreted in two ways. First, that cell adhesion to BSM was non-specific, due to charge attraction for example, or second, that adhesion arose via a specific molecular interaction with the haemocyte membrane, perhaps of a subpopulation of plasmatocytes which carry a not yet discovered sialic acid-specific lectin. Since sialic acid-containing glycoproteins are components of all animal cells it seems unlikely that haemocytes would carry sialic-acid specific lectins since these might recognise self. However, sialic acid-specific lectins have been found in other

invertebrate orders (Vasta et al., 1982a; Vasta and Marchalonis, 1985). Since an enormous diversity of antigens must be encountered during infection, to rely on only one glycosyl residue, D-galactose, for non-self recognition seems to present many loopholes. Perhaps then, other lectins with different sugar-binding characteristics occur on cockroach haemocytes conferring broader specificity and acuity of recognition. Indeed, the increase in BSM-adhering cells after infection with Hymenolepis (Fig. 5.3.1. and 5.3.2.) and injection with Zs (Fig. 5.2.2.) is difficult to explain if insect cells lack a sialic-acid specific lectin.

An alternative explanation for enhanced adhesion after immune stimulation might be that haemocytes from an activated immune system have a different surface charge to those from an unstimulated immune system. If this was the case, then charge repulsion between the cell and the glycoprotein-coated substratum may be altered so increasing the stickiness of the sialic acid-rich BSM for the haemocytes. The haemocytes of P. americana carry a net negative charge on their membranes (Takle and Lackie, 1985), although haemocyte populations are very heterogeneous with respect to surface negativity. It is possible then that altered adhesivity to BSM by haemocytes from immune stimulated animals may be due to enrichment of a less negatively charged cell subpopulation.

Sialic Acid - Does it affect Lectin Binding?

It has been proposed that sialic acids decrease the immunogenicity of glycoproteins by masking antigenic determinants (Horowitz and Pigman, 1977). However, this may be an oversimplification since sialic acids in BSM may participate as immunogens (Horowitz and Das, 1967).

In the present study, desialylation of PSM caused fewer cells to adhere in the flow chamber (Table 5.5.2) indicating either that the cells have some specificity for sialic acid or that desialylation alters the physical properties of the PSM molecule, either by affecting the molecular charge or by exposing other sub-terminal glycosyl residues. Since PSM has only 1% of bound sialic acid, desialylation seems unlikely to have a profound effect on the molecular structure. However, removing negatively-charged sialic acid residues is likely to reduce the negative charge on the glycoprotein bringing it nearer to neutrality. Since haemocytes adhere poorly to neutrally charged surfaces (Lackie, 1983; Chapter 4), it is possible that charge alterations are responsible for the decrease in adhesion to asialo-PSM.

Adhesion to desialylated BSM was higher than to native BSM (Fig./Table 5.5.1). This cannot readily be explained by charge interactions as for PSM, since after desialylation, the BSM molecule will carry a less negative charge to which fewer cells would be expected to adhere. However, since BSM carries much more bound sialic acid, removal of sialic acid is likely to have a greater effect on the properties of the BSM molecule compared with PSM. Removal of sialic acid by desialylation may unmask immunogens, such as sub-terminal D-galactose residues, to which the membrane galactose-specific lectin can then bind. Indeed, the sugar sequence proximal to the terminal sugar may also be important in lectin-binding specificity (Hardy et al., 1977). Vasta et al. (1982b) found that desialylated BSM efficiently inhibited vertebrate blood cell agglutination by a lectin in oyster serum. These authors proposed that exposure of D-galactose or N-acetyl-D-glucosamine in the desialylated BSM molecule was responsible for the

effect even though the haemocyte membrane has not been shown to have a lectin with this glycosyl specificity.

The Biological Significance of Lectin Binding

Since changes in cell adhesiveness underlie most of the cellular defence responses eg. nodule formation, encapsulation, it is important to find the biological significance of invertebrate lectin in the immune response. Evidence for a humoral agglutinating agent in the immune response of P. americana to a helminth was provided by Lackie (1981) who found that cockroach serum could agglutinate oncospheres of H. diminuta. However, little evidence exists to suggest that immune manipulation could affect cell recognition and adhesion by altering the lectin activity. Interestingly, Komano et al. (1980) have shown that the major serum lectin of the dipteran fly, Sarcophaga peregrina, is induced after wounding and during pupal development and Takahashi et al. (1986) found more lectin in Sarcophaga injected with foreign cells or bacteria compared with saline-injected controls. More recently inducible haemaglutinin activity has been detected in haemolymph of the silkworm, Bombyx mori, after infection with virus particles and may be attributable to lectin-like protein (Mori et al., 1989). However, the lectin of Hyalophora cecropia does not appear to be inducible since Castro et al. (1987) failed to raise the concentration of this lectin.

In the present study, 1 day after infection with H. diminuta (Fig. 5.3.1) the haemocytes became more adherent to the BSM-coated substratum and after 3 days cells adhered better to both glycoproteins. Improved adhesiveness was specific to the glycoproteins rather than an overall increase in stickiness since adhesion to BSA was unaffected.

These changes in adhesiveness may reflect enhanced expression of the

lectin on the cell surface and the findings of Takahashi et al. (1986) support this hypothesis. These authors reported that the serum lectin gene in fat body cells of Sarcophaga was activated and lectin production increased in response to cuticular wounding and foreign material. Alternatively, if more cells expressed the lectin then increased adhesion to the glycoproteins may be explained. Enrichment of the lectin-expressing cells could arise either by recruitment of haemocytes from resting sites or by the participation of other haemocyte subpopulations in the defence reaction. Whatever the nature of the enhanced adhesiveness, the final result is that more cells recognise surfaces which present galactose or sialic acid molecules.

The results for adhesion to asialo versus native BSM by haemocytes from Hymenolepis-stimulated animals are difficult to interpret. Hymenolepis infection reduces the adhesivity of cells for asialo-BSM compared with cells from naive animals and this does not support the hypothesis of exposed sub-terminal galactose in desialylated BSM acting as a recognition signal for adhesion. Further, in these experiments there is no change in adhesion to native BSM by blood cells from mature Hymenolepis-infected animals which does not agree with the results in Fig. 5.3.2.. These data suggest that cell adhesion to a substrata may be a more complex interaction which is dependent upon many variables.

If both Hymenolepis and Zs could activate a putative cellular recognition mechanism then does Moniliformis either suppress or evade this to survive in the haemocoel? Fig. 5.4.1, showing haemocytic adhesion after infection with Moniliformis acanthellae, would seem to suggest that all treatments lower the haemocytic adhesiveness to PSM. However, in this experiment the naive control values may be misleading

since comparison with other experiments suggests that the value is much higher than expected (see Results section) and statistical testing of the data shows that the control PSM-adhesion values are not significantly different from controls. Therefore, if the adhesion of haemocytes from control animals is unusually elevated because of wide experimental variations, then it seems that none of the treatments, including Moniliformis acanthellae infection, affect cell adhesion.

This has important implications for understanding the mechanism of parasite survival from immune attack since it can then be concluded that the acuity of haemocytic recognition is unaffected in acanthellae-infected animals. Therefore, immune evasion may occur by molecular mimicry of self or by the absence of recognisable carbohydrate moieties on the envelope surface. However, O'Brien (1988) suggested that the envelope of M. moniliformis may carry D-galactose residues, shown by binding with peanut agglutinin lectin. Since the major lectin of P. americana is galactose specific, cockroach haemocytes could potentially recognise and bind to the parasite sugar residues, although the specificity of peanut agglutinin is broader than for the cell lectin. Moreover, the evidence suggests that Moniliformis does not avoid immune attack by shedding immunogens to block lectin recognition sites since blood cells from infected animals readily adhere to both glycoproteins.

Interestingly, in cockroaches fed with Moniliformis acanthors (Fig. 5.4.2.), the haemocytic adhesion to PSM was slightly enhanced (all values non-significant), although the effect was low when compared with Hymenolepis infection. However, in contrast to Hymenolepis, Moniliformis acanthors did not encourage adhesion of haemocytes to BSM. Therefore, if improved adhesivity to the glycoproteins is used as an indicator of immune recognition and responsiveness, then the early

stages of Moniliformis development may have a slight stimulatory effect on the haemocytes.

In summary, it seems that cellular recognition may be mediated via a membrane-bound lectin with D-galactose specificity since adhesion of haemocytes to a galactose-rich glycoprotein (PSM) is greater than to a sialic acid-rich glycoprotein (BSM). Also, qualitative changes in cellular adhesivity appear to be indicative of immune reactivity since both Zs and Hymenolepis affected haemocyte adhesion to the glycoproteins. Interestingly, Moniliformis acanthors stimulate similar adhesive changes in the haemocytes which may reflect recognition by the host either of these early development stages or of gut damage associated with parasite penetration. Finally, it seems that established infections of Moniliformis do not adversely affect the ability of haemocytes to distinguish non-self surfaces via the lectin-ligand mechanism. These observations imply that Moniliformis acanthellae evade rather than actively reduce the acuity of recognition and that the efficiency of this survival mechanism improves as the parasite develops.

SUMMARY

1. Haemocyte adhesion has been studied *in vitro* under flow conditions to investigate the importance of the galactose-specific haemocyte membrane lectin in immune recognition and responsiveness.

2. Haemocytes from naive animals adhered well to galactose-rich glycoprotein (PSM) but poorly to sialic acid-rich and galactose-poor glycoprotein (BSM).

3. Fewer cells adhered to desialylated PSM compared to native PSM and the significance to the immune response of removing sialic acid from the PSM molecule has been discussed.

4. Adhesion to PSM and BSM after *in vivo* immune manipulation has been studied. Feeding with *Hymenolepis* proglottids increased adhesion to both glycoproteins. Zs injection enhanced adhesion to BSM only. *Moniliformis* acanthors caused a slight increase in cell adhesion to PSM whereas acanthellae had no effect on cell adhesion. From these results, it has been concluded that *Hymenolepis* is highly stimulatory to the cockroach immune system whereas Zs and *Moniliformis* acanthors are less stimulatory and *Moniliformis* acanthellae are neither stimulatory or inhibitory to cell adhesion.

5. It is proposed that cell recognition is mediated via the membrane bound lectin and that qualitative changes in cell adhesion indicate immune reactivity. Finally, these results provide evidence for immune evasion by *Moniliformis* acanthellae being the survival mechanism for this parasite in the intermediate host.

CHAPTER 6 - CYTOCHEMISTRY

CHAPTER 6 - CYTOCHEMISTRY

INTRODUCTION

Identification of Haemocyte Subpopulations using Cytochemistry

The plasmatocytes of *P. americana* play a major role in phagocytosis (see Chapter 3) and encapsulation (see Chapter 4; Lackie *et al.*, 1985) and are a behaviourally heterogeneous population (Takle and Lackie, 1986). The haemocytes of several invertebrates have been successfully separated (Soderhall and Smith, 1983; Smith and Soderhall, 1983b ; Mead *et al.*, 1986; Huxham and Lackie, 1988), providing researchers with the many advantages of working with pure or monoclonal antibody identified (Dikkeboom *et al.*, 1988) cell populations. However, the haemocytes of *P. americana* have proved difficult to separate physically on Percoll gradients (Huxham, pers. comm.) and monoclonal antibodies directed against *P. americana* haemocyte classes do not yet exist. Although haemocyte classes can be distinguished on their morphology and ultrastructure, this provides little information on their function in the immune response.

In this chapter, cytochemical methods have been used to distinguish haemocyte subpopulations by identifying and quantifying granule-associated enzymes in plasmatocytes.

Lysosomal Enzymes - Susceptibility and Resistance to Infection

Granath and Yoshino, (1983) and Dikkeboom *et al.* (1984) used cytochemical staining of molluscan haemocytes to characterise several blood cell subpopulations. Moreover, these authors attributed vector susceptibility or resistance to infection to the relative abundance of

the intracellular lysosomal enzymes.

Since lysosomal enzymes appear, at least in part, to be responsible for resistance to infection in other invertebrates, by studying the cytochemistry of haemocytes from infected cockroaches some questions on the immune responsiveness and on host susceptibility (Moniliformis) or resistance (Hymenolepis) to infection may be answered. In this chapter, the enzyme content of circulating plasmatocytes from naive and infected cockroaches has been investigated. Since lysosomal enzymes play a role in intracellular digestion and killing, peroxidase and acid phosphatase were studied.

Peroxidase and Intracellular Killing

An increase in the metabolic activity, called the respiratory burst, accompanies phagocytic uptake in mammalian polymorphonuclear leucocytes. Oxygen consumption increases and, through a complex series of enzymic reactions, hydrogen peroxide (H_2O_2) is evolved. Toxic oxygen metabolites are also produced and these include the hydroxyl radical (.OH), singlet oxygen ($^1O_2$) and the superoxide anion (O_2^-) (see review by Klebanoff et al., 1983).

The hydrogen peroxide alone is toxic to microbes, but in the presence of myeloperoxidase and a halide, the effect is enhanced. The myeloperoxidase-halide-hydrogen peroxide anti-bacterial system (Klebanoff, 1968) is important in bacterial killing, since humans suffering metabolic defects eg. chronic granulomatous disease frequently suffer chronic bacterial infections (Holmes et al., 1967; Holmes and Good, 1972).

The process of immune killing, both intracellular and extracellular, is well understood in the vertebrates compared with the invertebrates.

The evidence for the respiratory burst and associated enzymes (eg. peroxidase) in the defence reactions of invertebrates is equivocal and biased towards the molluscs. Cheng (1976) concluded that the myeloperoxidase-hydrogen peroxide-halide anti-microbial system was absent in haemocytes of the clam, Mercenaria mercenaria since, during phagocytosis of heat-killed bacteria, oxygen consumption did not increase and hydrogen peroxide was not found in the cells. McKerrow and co-workers (1985) failed to detect peroxidase activity in snail haemocytes. However, recent evidence (Dikkeboom et al., 1984; 1987) suggests respiratory burst activity in phagocytosing molluscan haemocytes. Nitroblue tetrazolium (NBT) reduction, identified by a blue deposit (Cagan and Karnovsky, 1964), indicated sites of superoxide evolution in the snail haemocytes (Dikkeboom et al., 1988).

Evidence suggests that insect haemocytes do not have a comparable anti-microbial system (Anderson, 1974; Anderson et al. 1973), yet intracellular killing and digestion are efficient. This lack of knowledge is unfortunate in view of the importance of insects as parasite vectors. Only by first understanding how invaders are destroyed can we hope to interfere with the host-parasite interaction, with the aim of making previously susceptible hosts resistant to infection. With this in mind, cytochemical methods were used to investigate whether peroxidase and superoxide, components of the mammalian respiratory burst system, are present in the haemocytes of P. americana.

Acid Phosphatase and Digestion

The enzymes and toxic metabolites of the respiratory burst may work

alone to kill the phagocytosed or encapsulated organisms, or they may work in combination with the lysosomal enzymes eg. acid phosphatase. Acid phosphatase activity appears to be ubiquitous in haemocytes of the higher invertebrates being reported frequently in molluscs (Huffman and Tripp, 1982; Granath and Yoshino, 1983; Dikkeboom et al., 1984; McKerrow et al., 1985; Cheng and Mohandas, 1985; Yoshino and Cheng, 1976; Franchini and Ottaviani, 1990), crustaceans (Hose et al., 1987) and insects (Chain and Anderson, 1983b; Rowley and Ratcliffe, 1979). Several authors (Cheng and Butler, 1979; Cheng and Mohandas, 1985; Cooper-Willis, 1979) have found increased levels of haemolymph acid phosphatase and amino-peptidase (Mohandas and Cheng, 1985) after immune stimulation suggesting that enzymes released from haemocytes may participate in the immune response.

Inducible Anti-bacterial Activity

Lysozyme was the first immune protein to be isolated and characterised in the silk moth Bombyx mori (Powning and Davidson, 1973; 1976) and has been found in the haemolymph of many insect species (Mohrig and Messner, 1968). Lysozyme is inducible and increased haemolymph activity has been found after wounding, injection with latex particles, bacterial LPS (Anderson and Cook, 1979) and whole bacteria (Hoffman and Brehelin, 1976; Kaaya and Darji, 1988) and after infection with protozoan parasites (Ingram et al., 1984). This enzyme has also been detected in insect haemocytes (Anderson and Cook, 1979; Zachary and Hoffman, 1984; Trenczek, 1988).

The aim of the work reported in this chapter was to identify subpopulations of plasmatocytes using immunohistochemical and cytochemical staining and to investigate the distribution and abundance

of the enzymes after infection. This information may indicate functions for different plasmatocyte subpopulations in the immune response and the overall level of immunocompetence in Hymenolepis and Moniliformis-infected animals. Finally, since little is known of intracellular killing in insects any information on this aspect of the immune response might be important for biological control of insect populations.

RESULTS

6.1. IMMUNOCHEMISTRY

Lysozyme

Using indirect immunofluorescence, around 40% of cockroach haemocytes contained fluorescent granules. The granules varied in size and were distributed throughout the cytoplasm, although more often perinuclear. Stained cells were characteristically large, well spread and contained other unstained granules. The most intensely stained cells were round, similar to unspread plasmatocytes. Lysozyme negative cells were usually spindle-shaped and polarised with the ruffled leading membrane typical of locomoting cells.

6.2. CYTOCHEMISTRY

Acid Phosphatase

Using the azo-dye method (Pearse, 1968), the blue reaction product indicated the sites of acid phosphatase activity in the cytoplasm of the haemocytes. Sodium fluoride completely inhibited the acid phosphatase reaction. All other controls were negative. The stain was located in perinuclear granules and these are lysosomes, since acid phosphatase is a lysosomal marker.

The enzyme was present in both the plasmatocytes and the coagulocytes. Around 80-90% of plasmatocytes contained acid phosphatase and all coagulocytes were stained. The intensity of staining in the plasmatocytes was highly variable, depending on the number of granules. In addition, the granules were highly variable in size. A small percentage of cells contained large strongly positive granules. These

granules were identified as secondary lysosomes since they also contained phagocytosed latex particles (Plate 6.2.a). Similar findings have been reported by Rowley and Ratcliffe (1979) in plasmatocytes of Galleria mellonella phagocytosing latex particles.

Peroxidase

The results are shown on Plate 6.2.a-b.

Haemocytes were stained for peroxidase using the DAB method of Graham and Karnovsky (1966). Dark brown deposits of reaction product around the nucleus indicated peroxidase activity. Peroxidase was present in approximately 20-30% of plasmatocytes and was absent from coagulocytes. Activity was perinuclear and there was no activity in the cytoplasmic extensions of spread plasmatocytes. Peroxidase was located in granules of variable size.

Plasmatocytes often contained different numbers of granules, from several hundreds to fewer than 10. Peroxidase was not found in cells which had phagocytosed latex particles. The peroxidatic activity was abolished when the substrate (DAB) was omitted from the reaction mixture. Omission of hydrogen peroxide from the reaction had little effect on the staining efficiency indicating that hydrogen peroxide is available endogenously. The catalase inhibitor, aminotriazole, did not affect the reaction so catalase was not responsible for the reaction product.

6.2.1. Zymosan Supernatant Injection

In this series of experiments, the effect of stimulatory molecules (Zs) on the distribution and quantity of intracellular enzymes was studied.

Haemocytes contained both peroxidase and acid phosphatase.

a. Peroxidase

The results are shown in Fig. 6.2.1.a.

Analysis of the pooled data using oneway analysis of variance revealed that treatment with either saline or Zs significantly reduced the total percentage of peroxidase stained cells ($F=4.25$, d.f.=2,60, $p<0.05$). A significant decrease ($F=5.50$, d.f=2,60, $p<0.01$) in the percentage of highly stained cells was largely responsible for the overall changes in peroxidase in cells from saline and Zs treated animals.

The overall total proportion of peroxidase stained cells had returned to control levels 12hr after treatment (Fig. 6.2.1.a). This occurred in all of the experimental replicates, although in Exp. 2 the percentage of weakly stained cells was significantly elevated ($F=5.05$, d.f=2,19, $p<0.05$).

b. Acid Phosphatase

The results are shown on Fig. 6.2.1.b.

Comparison of the pooled results implied that acid phosphatase staining in haemocytes was unaltered by saline or Zs injections 3hr before cell collection. However, in Exp. 2, a significant reduction in the percentage of cells stained for acid phosphatase occurred after saline injection. This may be a real effect but it could be caused by inadequate cover of the monolayer with the reagents.

Twelve hours after treatment, acid phosphatase was significantly reduced ($F=6.1$, d.f.=2,57, $p<0.01$) in saline injected controls compared with naive and Zs injected animals. The overall change in saline controls resulted from low numbers of intensely stained cells ($F=7.01$,

d.f.=2,57, $p<0.01$).

6.2.2. Infection with H. diminuta

a. Peroxidase

The results are graphed in Figures 6.2.2.a.

The most important feature of the results is the elevation in the levels of peroxidase in the cells from insects fed viable proglottids compared with controls. This effect is highest three days after feeding with the parasite, although there seems to be a gradual increase over the preceding days. A significantly higher proportion ($F=3.57$, d.f.=2,55, $p<0.05$) of the cells from one day Hymenolepis-infected insects contained peroxidase compared with control insects. An increase ($F=3.92$, d.f.=2,55, $p<0.05$) in the proportion of darkly stained cells was found to be responsible for the overall increase.

b. Acid Phosphatase

Fig. 6.2.2.b shows the percentage of acid phosphatase positive cells in the haemolymph of naive and Hymenolepis-fed cockroaches. There is no significant change in the total proportion of stained cells in the animals either 1 day ($F=0.05$, d.f.=2,60, $p>0.05$) or 3days ($F=2.3$, d.f.=2,59, $p>0.05$) after feeding with the parasite, when the results for replicates were pooled. However, analysis of individual replicates reveals that there are inconsistencies between experiments. In Exps. 1 and 3, on day 1 after feeding with viable oncospheres, there is a significant reduction in the number of intensely stained cells which is sufficient in both experiments to reduce significantly the overall number of stained cells compared with naive animals. In contrast, in

Exp. 2, the acid phosphatase levels are much higher in animals fed viable proglottid, although this does not have a marked effect on the total percentage of cells with acid phosphatase.

A similar picture emerges when the results for the three day replicates are analysed. In Exp. 1, there is a higher percentage of cells containing large amounts of acid phosphatase in the parasite-fed animals than in the naive animals but, as above, this is insignificant when the results for the total percentage of acid phosphatase-stained cells in the experiment are analysed. In Exp. 3, the total percentage of stained cells is significantly reduced after feeding with Hymenolepis ($F=4.66$, d.f.=2,18, $p<0.05$). This is caused by a decrease in both the weakly and the intensely stained cells in the circulating cell population. These differences between experiments suggest that other variables eg. crop emptying rates, are contributing to acid phosphatase levels in the cells and that the situation is one which will not readily be resolved.

6.2.3. Infection with M. moniliformis

a. Peroxidase

Acanthellae of Moniliformis in the haemocoel of cockroaches caused a highly significant reduction ($F=4.22$, d.f.=3,73, $p<0.01$) in the proportion of peroxidase positive cells in the circulating cell population (see Fig. 6.2.3.a). Sections of ovariole also promoted a small, but not significant, decline in the peroxidase-containing cell number compared with the naive and medium-injected controls. A decrease in both the intensely and the weakly stained cell population was responsible for the observed change in the total stained cells. In Exp.

3, the significant decline in the total proportion of plasmatocytes stained for peroxidase was due to a large reduction in the numbers of intensely stained cells rather than the weakly stained cells.

6.2.4. Superoxide Production

The metabolic activity of phagocytes can be detected by assaying the reduction of nitroblue tetrazolium (NBT) to formazan (Badwey et al., 1986). Blue deposits of formazan indicated the sites of superoxide evolution. All cells were lightly stained although some were more intensely stained than others. Stained granules were small and distributed throughout the cytoplasm. Cell aggregates, or nodules, contained most reaction product. This was probably an artifact arising from the number of cells forming the nodule. Where phagocytosis had occurred, there was no change in the intensity of stain associated with the secondary lysosome and the number of particles phagocytosed did not affect the intensity of staining. Incubation time and particle concentration did not alter the staining, and haemocytes from each individual exhibited the same degree of superoxide production.

In control experiments, superoxide dismutase (SOD) had no effect on

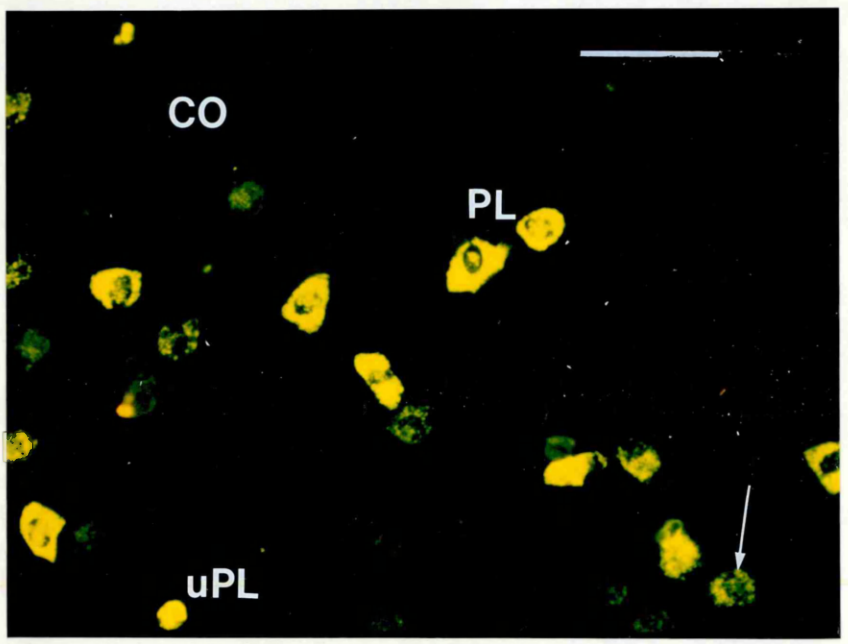
phagocytic uptake by the haemocytes or on the ability of cells to spread. Furthermore, there was no change in the staining pattern of the cells after preincubation with SOD.

Plates 6.1.a-b

Fluorescence micrographs of P. americana haemocytes indirectly immunolabelled for insect lysozyme. Note highly granular stain in plasmatocytes. Scale bar = 100μm

PL=plasmatocyte, uPL=unspread plasmatocyte, CO=coagulocyte, arrows=granules

a. x250 magnification



b. x550 magnification. Scale bar = 40μm

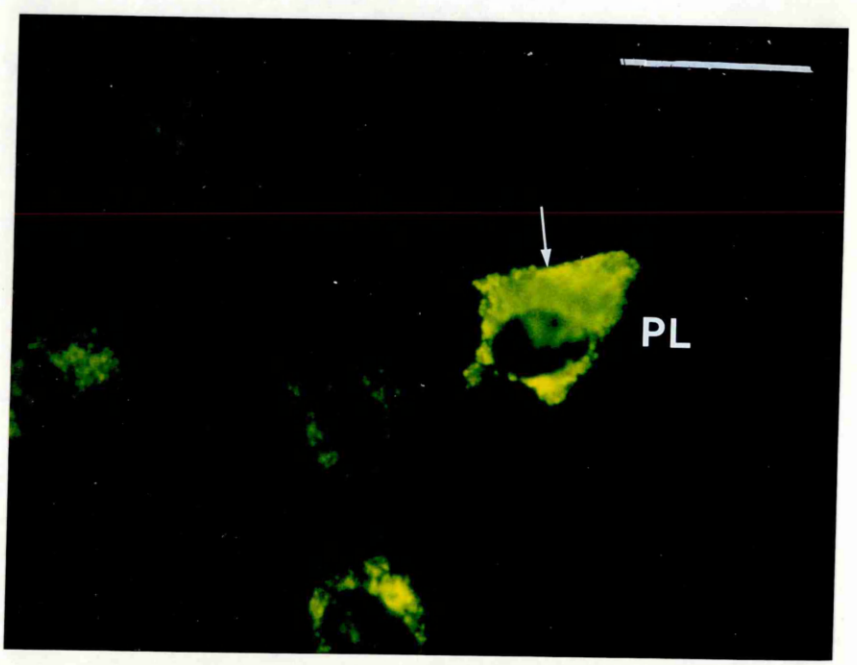
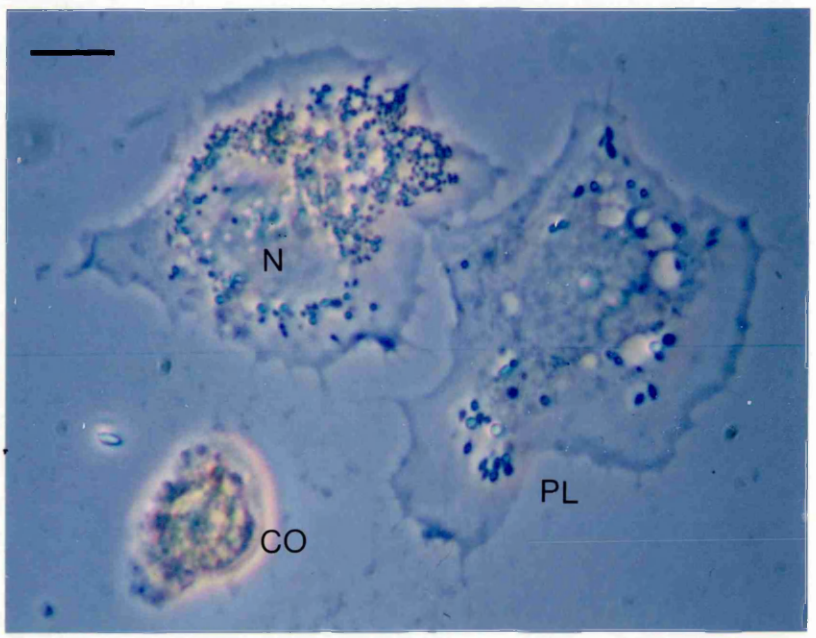


Plate 6.2.a-b

Light micrographs of haemocytes from P. americana showing intracellular peroxidase reaction product. Scale bar = 5µm

PL=plasmatocyte, CO=coagulocyte, N=nucleus, arrows=dark brown reaction product.

a. Phase contrast micrograph showing morphology of stained cells.



b. Light micrograph of the same cells in 6.2.a showing peroxidase staining.

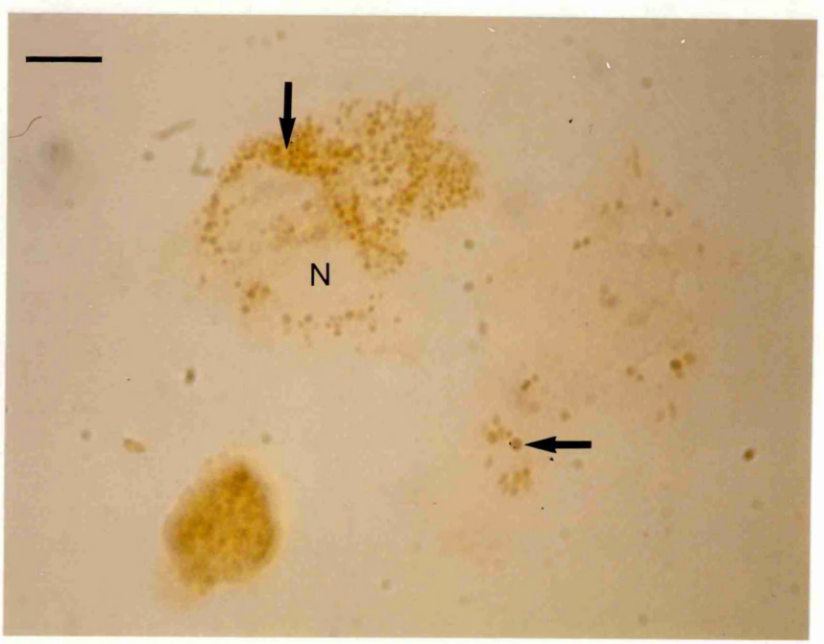
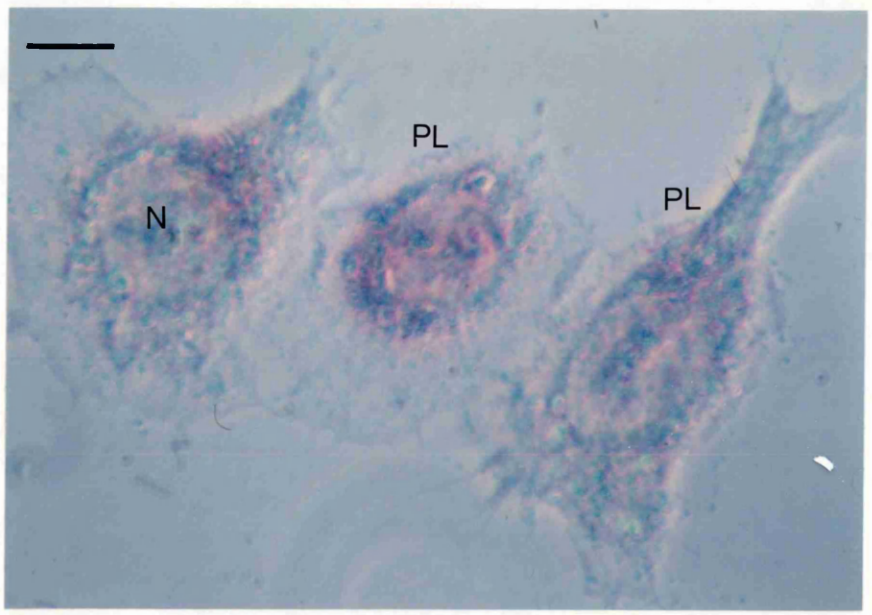


Plate 6.2.c-d

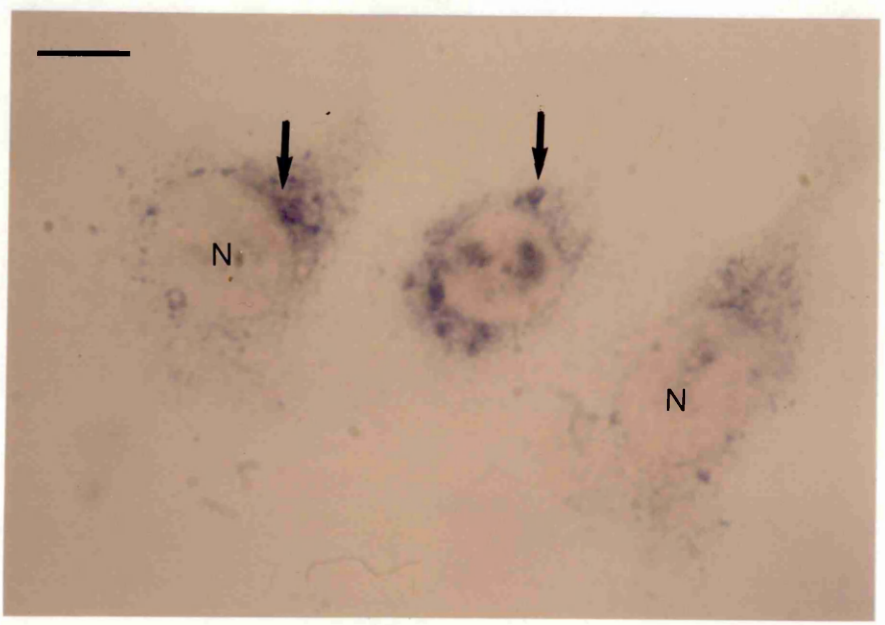
Haemocytes from P. americana showing intracellular acid phosphatase reaction product. Scale bar = 5µm.

PL=plasmatocyte, CO=coagulocyte, N=nucleus, arrows=blue reaction product.

c. Phase contrast micrograph showing morphology of stained cells.



d. Light micrograph of the same cells in 6.2.c showing staining pattern for acid phosphatase.



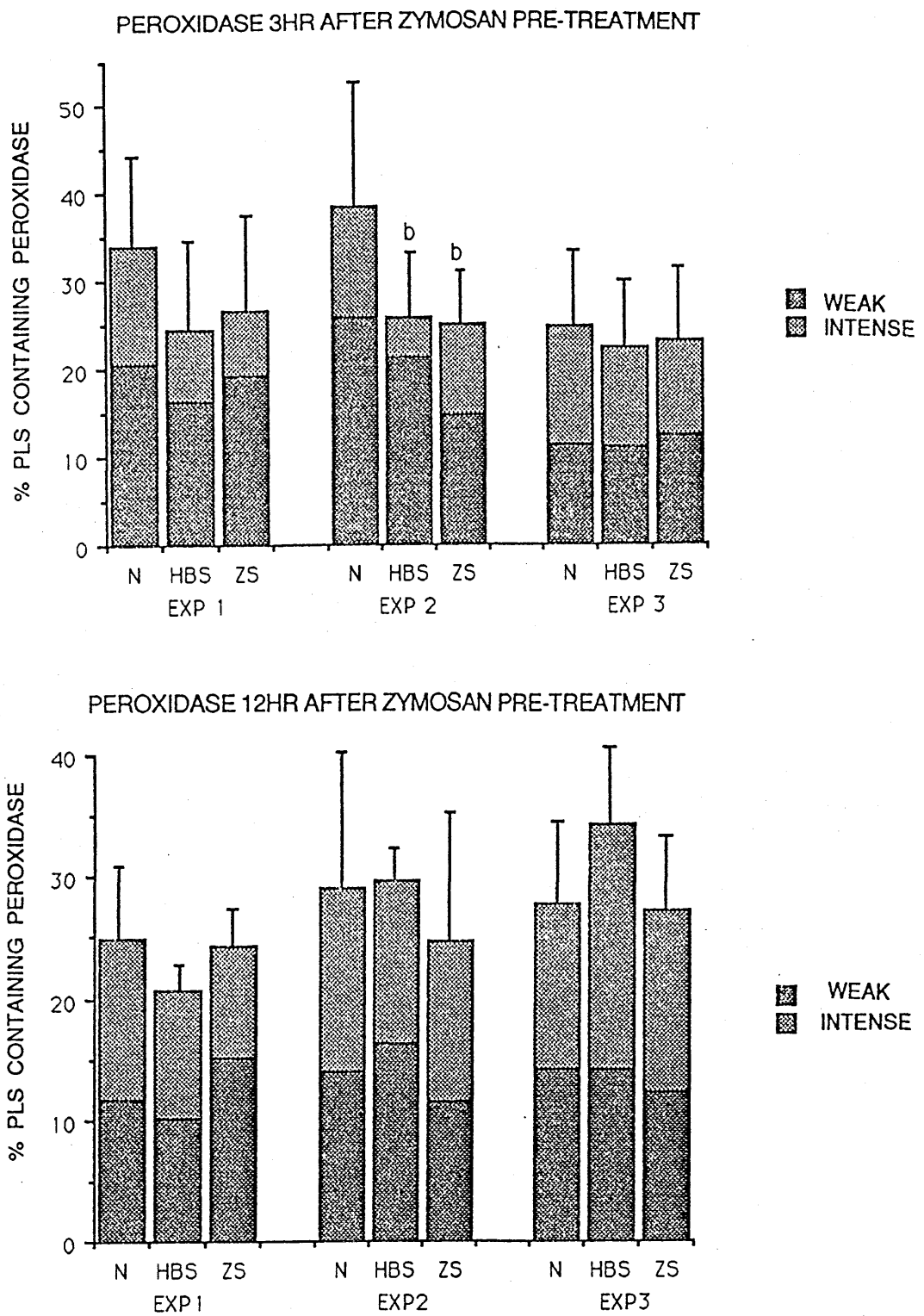


Figure 6.2.1.a
Presence of peroxidase in haemocytes from adult male Periplaneta americana injected with Zymosan A supernatant 3 or 12hr previously. Results are expressed as a mean % ± s.d. plasmatocytes counted containing enzyme (> 300 cells counted / animal). Data from 7 animals / treatment, except where stated. N = naive, HBS=saline injected, Zs=Zymosan supernatant injected.

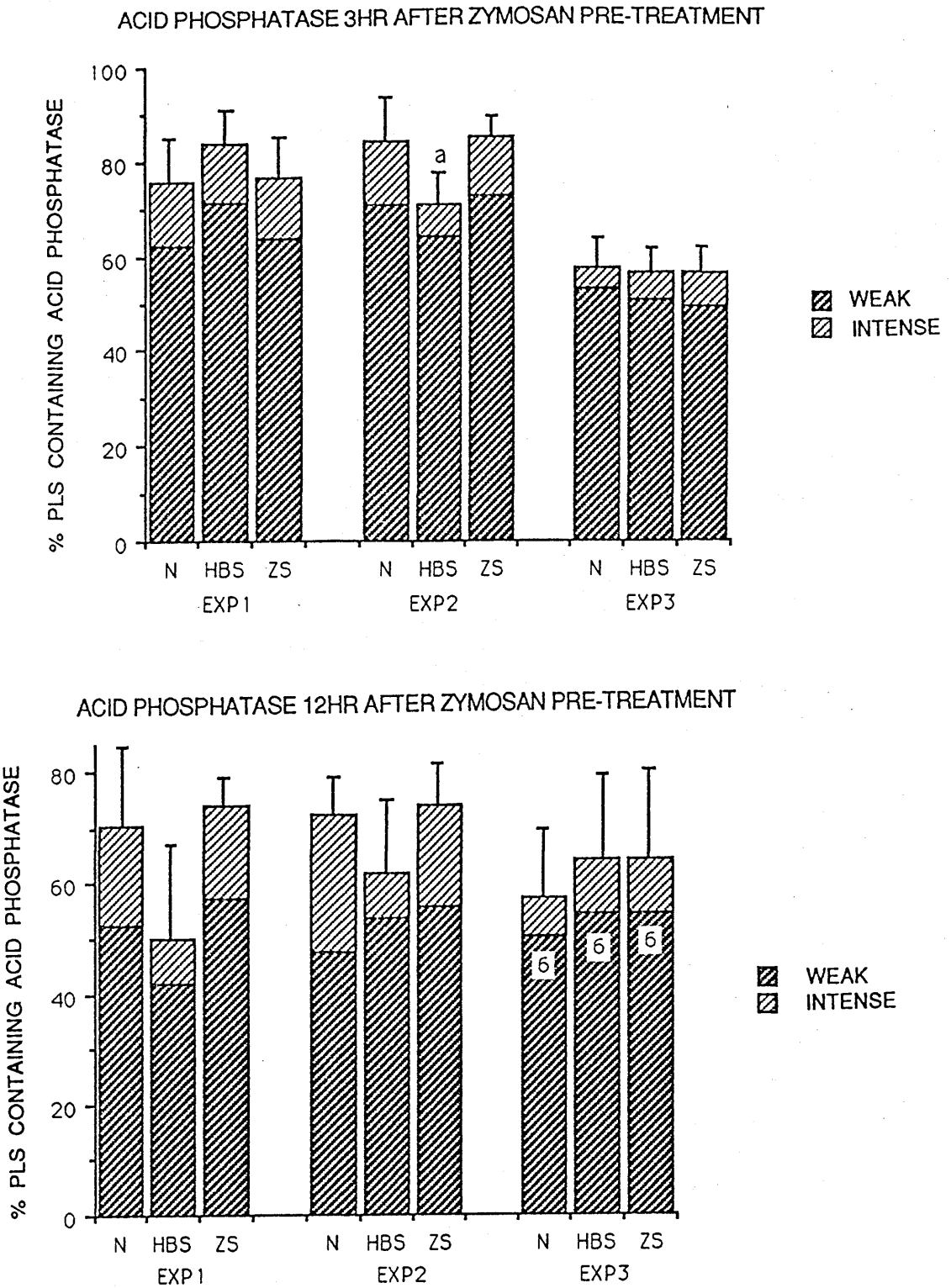


Figure 6.2.1.b
Presence of acid phosphatase in haemocytes from adult male Periplaneta americana injected with Zymosan A supernatant 3 or 12hr previously. Results are expressed as a mean % ± s.d. plasmatocytes counted containing enzyme (> 300 cells counted / animal). Data from 7 animals / treatment, except where stated. N = naive, HBS = saline injected, Zs = Zymosan supernatant injected.

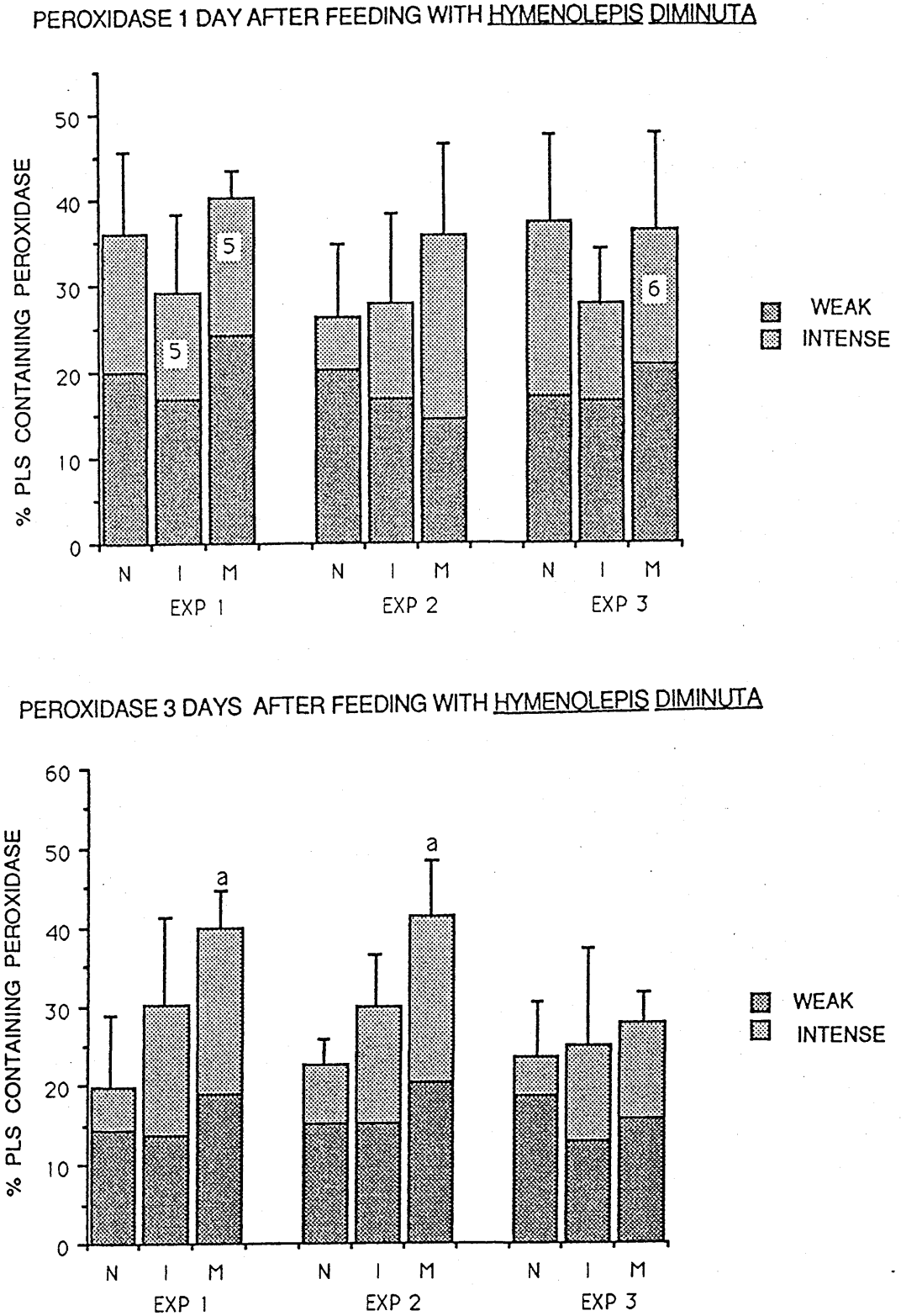
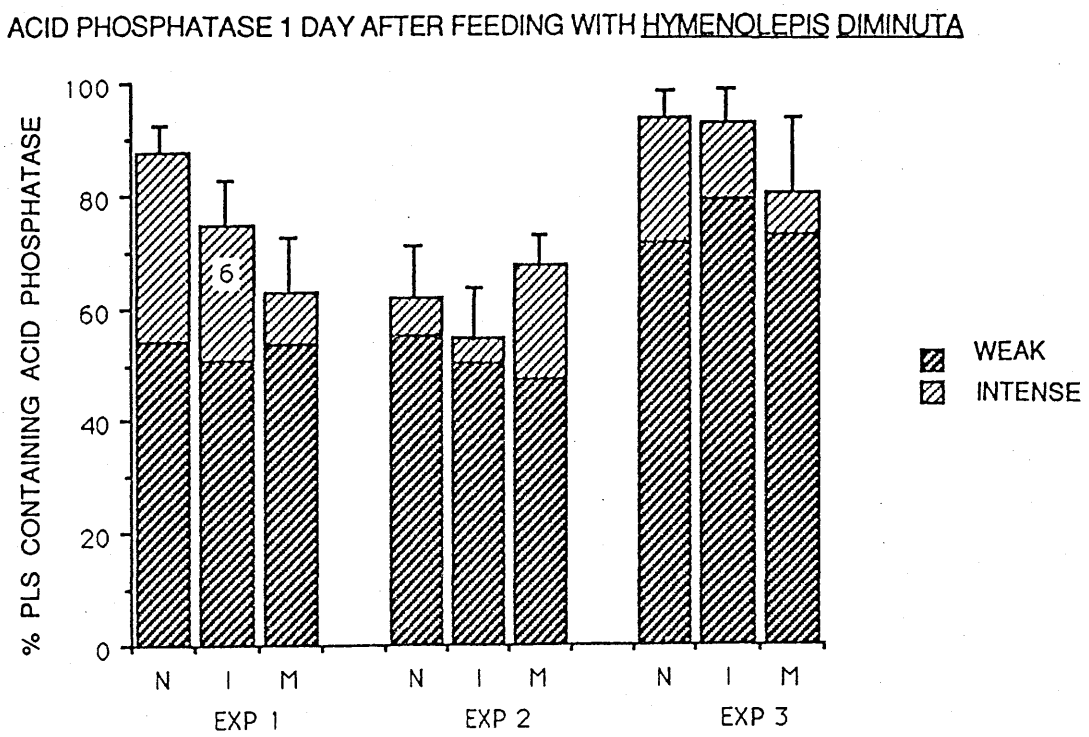


Figure 6.2.2.a
Presence of peroxidase in haemocytes from adult male Periplaneta americana fed Hymenolepis diminuta 1 or 3 days previously. Results are expressed as a mean % ± s.d. plasmatocytes counted containing enzyme (> 300 cells counted / animal). Data from 7 animals / treatment, except where stated. N = naive, I = immature Hymenolepis, M = mature Hymenolepis.



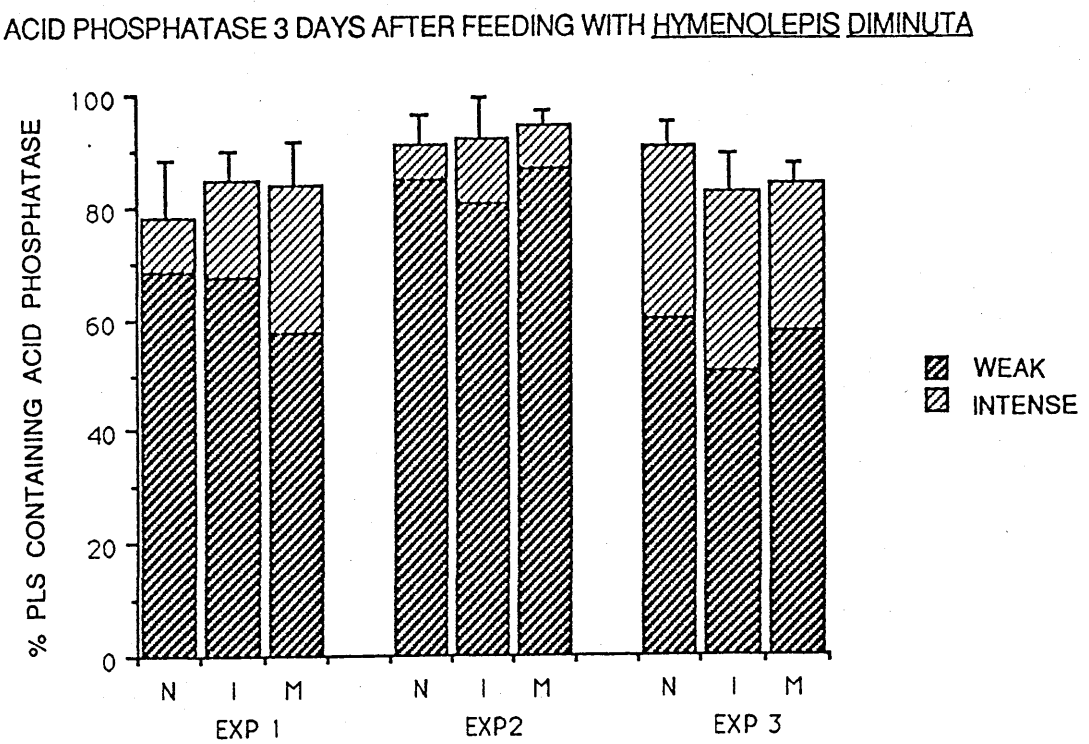


Figure 6.2.2.b
Presence of acid phosphatase in haemocytes from adult male Periplaneta americana fed Hymenolepis diminuta 1 or 3 days previously. Results are expressed as a mean % ± s.d. plasmatocytes counted containing enzyme(300 cells counted / animal). Data from 7 animals / treatment, except where stated. N = naive, I = immature Hymenolepis, M = mature Hymenolepis.

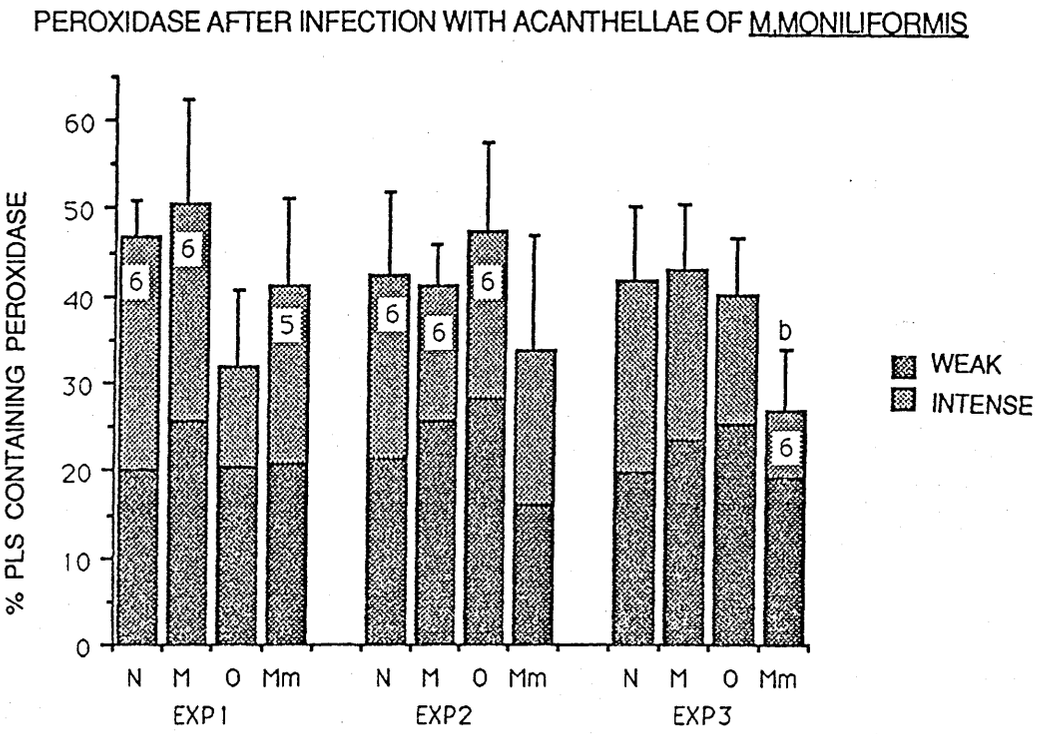


Figure 6.2.3.
Presence of peroxidase in haemocytes from adult male Periplaneta americana infected with acanthellae II of Moniliformis moniliformis 8 days previously. Results are expressed as a mean % ± s.d. plasmatocytes counted containing enzyme (> 300 cells counted / animal). Data from 7 animals / treatment, except where stated. N = naive, M = medium injected, O = ovariole transplant, Mm = Moniliformis acanthellae (40).

DISCUSSION

Using cytochemical staining techniques, acid phosphatase, peroxidase and lysozyme have been found in the granules of plasmatocytes of P. americana.

Acid phosphatase is a lysosomal acid hydrolase which can be used as marker for these organelles. This enzyme has been visualised in the phagosome of phagocytic plasmatocytes of P. americana indicating that the lysosomal enzymes are involved in intracellular digestion of phagocytosed material in cockroach blood cells. Similar findings have been reported for haemocytes of Galleria mellonella (Ratcliffe and Rowley, 1979). Immunohistochemical studies of the cockroach haemocytes revealed that lysozyme and attacin, but not P4, were present in the plasmatocytes. These enzymes have been found in haemocytes from Hyalophora cecropia (Trenczek, 1988).

The Killing Mechanism

Although acid phosphatase and lysozyme are likely to be involved in degradation of ingested material, the mechanism by which insect cells kill ingested or encapsulated material is unclear. Vertebrate phagocytes kill ingested bacteria using the highly efficient myeloperoxidase system in which toxic oxygen metabolites are evolved (for review see Klebanoff et al., 1983). The results from the NBT experiments confirm previous reports that insect haemocytes do not respond to infection by respiratory burst activity (Anderson et al., 1973; Anderson, 1976). Although the haemocytes stained lightly with NBT, this was neither inhibited by SOD or increased by phagocytosis of zymosan particles. It seems then that increased evolution of superoxide

does not occur in insect cells. This is in agreement with chemiluminescence studies of these cells, in which Zs did not stimulate a detectable respiratory burst response (Huxham and Lackie, unpubl. obs.).

However, peroxidase activity was detected in the plasmatocytes in the present study and Misko (1972) also reported histochemically-located peroxidase in P. americana. Since the evidence is that there is no respiratory burst in insect haemocytes, it is important to consider whether the peroxidase activity is due to another intracellular enzyme. Phenoloxidases, which are involved in sclerotisation in the cuticle and melanisation of capsules (for review see Brunet, 1980; Anderson, 1985), are contenders for several reasons. First, it is found in the granules of plasmatocytes from P. americana (Huxham, unpubl. obs) and, second, it exhibits wide substrate specificity (Sugamaran, 1988). In a recent paper, Binnington and Barrett (1988) have shown that laccases which are a subtype of phenoloxidase react with diaminobenzidine (DAB), the substrate used to assay peroxidase activity. Laccases are found in the cuticle but not the haemocytes of Lucilia cuprina. These authors also detected cuticular staining with DAB even in the absence of hydrogen peroxide suggesting that peroxidase is not responsible for the end-product. Similarly in the present study, haemocytes stained well in the absence of hydrogen peroxide.

Further evidence for the enzyme assay detecting phenoloxidase rather than peroxidase is provided by comparing the proportions of haemocytes staining. Approximately 30% of haemocytes stain in vitro for peroxidase which is higher than the percentage of cells staining for phenoloxidase (Huxham et al., 1989). However, these differences can be explained on examination of the cell culture conditions. These authors culture cells

with FCS which is known to reduce the extent of pro-phenoloxidase activation in haemocyte lysates (Huxham, pers.comm.) and therefore, interfere with phenoloxidase production. Finally, Hymenolepis stimulated an increase in the peroxidase staining of the cells and this parasite also increases phenoloxidase production (Holt, 1989a). If, as discussed above, the assay detects phenoloxidase rather than peroxidase, then the reaction product found in capsules (see Chapter 4) could represent sites of phenoloxidase activity.

Contrary evidence for the peroxidase-phenoloxidase hypothesis is that neither Zs, containing β1,3-glucans, or infection with Moniliformis affected peroxidase staining. Zymosan supernatant activates prophenoloxidase components in cockroach haemocyte lysates (Dularay and Lackie, 1985; Takle, 1986; own observations) and phenoloxidase-positive aggregates are less common in haemocytes from Moniliformis-infected individuals (Lackie and Holt, 1988). With this in mind, it seems possible that the assay does not detect phenoloxidase. However, phenoloxidase activity is detected in the above assays by incubation with DOPA which is the preferred substrate for tyrosinases but not laccases. It may be then that Zs can stimulate tyrosinase activity but not laccase activity. Binnington and Barrett (1988) have shown quite clearly that these two enzymes are quite distinct in their induction.

Further evidence for peroxidase activity rather than phenoloxidase (laccase) is that the activity of the latter is not preserved by glutaraldehyde fixation (Binnington and Barrett, 1988) whereas in the present study, glutaraldehyde proved to be a suitable fixative for the peroxidase-like activity.

This problem of enzyme identification may be clarified by co-

incubation with phenylthiourea, a potent inhibitor of melanisation. Tyrosinases have been shown to be inhibited with phenylthiourea whereas laccases are not (Binnington and Barrett, 1988). Another approach to differentiating between the phenoloxidase-like activity (tyrosinase) and the peroxidase-like activity (laccase) might be immunohistochemical staining with phenoloxidase antibody (Ashida et al., 1988; Binnington and Barrett, 1988) prior to peroxidase staining.

If the assay does detect peroxidase rather than a subtype of phenoloxidase, it may play some other role in immune defence aside from the respiratory burst. The phagocytic subpopulation of plasmatocytes did not contain peroxidase activity, so this enzyme is most likely not involved in intracellular killing of phagocytosed material. However, peroxidase may perform some other function in immune defence, for example in the extracellular killing of encapsulated invaders. Peroxidase has recently been found in the cuticle of insects (Sugamaran, 1988) and is located in the granules of epidermal cells from Calpodes ethlius during cuticular hardening and tanning (Locke, 1969). Cuticular peroxidase has been found to be active without hydrogen peroxide (Locke, 1969) and it has been suggested that laccases act as an oxidising system for peroxidase activity (Binnington and Barrett, 1988). From the preceding evidence, it seems possible that the haemocytes do contain peroxidase and the question of biological function for this enzyme arises.

Sugamaran (1988) concluded from in vitro studies that peroxidase is involved in cuticular quinone tanning. Since quinones may be toxic to live material (Poinar et al., 1979), haemocytic peroxidase may kill invaders by quinone production in capsules, rather than by toxic oxygen evolution, as in the respiratory burst.

In summary, it appears that the respiratory burst (NBT reduction) does not occur in cockroach cells although peroxidase-like activity (DAB staining) is present in the haemocytes and capsules from this animal. For practical purposes in this discussion the term "peroxidase" will be used forthwith to indicate the peroxidase-like activity detected in the cells.

Haemocyte Subpopulations

Several plasmatocyte subpopulations have been distinguished using cytochemical staining. In naive cockroaches approximately 90% of plasmatocytes stain for acid phosphatase, 30-35% contain peroxidase and around 30% are positive for lysozyme, although the stained cells exhibited identical morphology. These results show that the acid phosphatase subpopulation must contain both peroxidase and lysozyme and is likely, therefore, to be heterogeneous with respect to function.

It is not possible to define further the plasmatocyte subpopulations since repeated attempts at immunohistochemical (lysozyme) and cytochemical (acid phosphatase or peroxidase) co-staining were unsuccessful due to problems with inadequate fixation. However, the phagocytic plasmatocyte subpopulation (5-10%) contains acid phosphatase but not peroxidase and proves that the plasmatocytes are indeed heterogeneous in function.

All stained subpopulations exhibit variation in the intensity of staining. This may represent different developmental stages of the haemocytes, the weakly stained cells being less differentiated immature cells, but other explanations are possible, such as the extent of enzyme storage or release into the haemolymph. Indeed, serum acid

phosphatase in Biomphalaria glabrata is elevated after challenge with live (Cheng and Butler, 1979) and heat-killed Bacillus megaterium which may be due to lysosomal release from intact cells (Cheng and Mohandas, 1985). However, elevated serum enzyme activity could also be caused by cell lysis because of immune stimulation. Since acid phosphatase has an optimum activity around pH5, the functional significance of acid phosphatase release into the haemolymph is doubtful.

Cytochemistry and Immune Stimulation

Immunocompetence and susceptibility to infection may be partially dependent on the lysosomal enzyme content of the blood cells. Juvenile snails of Lymnaea stagnalis have lower peroxidase levels and are more susceptible to infection than adult snails (Dikkeboom et al., 1984) and schistosome-resistant strains of B. glabrata have a much higher haemocytic enzyme content (peroxidase, acid phosphatase and non-specific esterase) than susceptible strains (Granath and Yoshino, 1983). Differences in the haemocyte phenoloxidase activity of mosquito strains has recently been reported as being relevant to the success of the mosquitoes in destroying microfilariae of Dirofilaria immitus (Li et al., 1989).

In the present work the enzyme constituents of the cellular immune system were altered as a specific response to parasitism rather than an inherent characteristic of the host defence system. Hymenolepis enhanced the immunocompetence of the immune system since there were considerably more peroxidase-positive cells after infection and these contained more enzyme than in uninfected individuals. Since Hymenolepis also increases the circulating cell number of infected cockroaches (chapter 3), the total peroxidase activity within the

immune system of Hymenolepis-infected animals is much higher than in uninfected individuals. A similar induction of enzyme activity (phenoloxidase) has been detected in microfilariae-inoculated mosquitoes compared with controls (Li et al., 1989).

Surprisingly, Zs did not induce changes in the haemocyte enzyme content. This suggests that β1,3-glucans are not sufficient stimulant to induce changes in the later stages of the immune response ie. melanisation and/or killing. Furthermore, since the THC is reduced after stimulation with Zs, the overall enzyme content of the immune system is reduced after Zs treatment.

In contrast with Hymenolepis, the enzyme content (peroxidase and acid phosphatase) of the immune system is unaffected by Moniliformis infection. This shows that it is not a lack in the destructive machinery (acid phosphatase and peroxidase) of the cells which permits parasite survival. Selective inhibition of the enzymes could be sufficient for Moniliformis survival yet still leave a responsive immune system capable of preventing other opportunistic infections. Phenoloxidase activity is known to be reduced in parasitised animals (Lackie and Holt, 1988) and partial or even complete inhibition of phenoloxidase activity has been detected in host lepidopterans infected with different species of parasitoid wasps or purified parasitoid virus particles (Stoltz and Guzo, 1983). Similarly, Beckage and her colleagues (1990) have recently reported that haemolymph phenoloxidase activity is depressed in Manduca sexta larvae parasitised with the braconid wasp, Cotesia congregata and polydnavirus particles. Inhibition of phenoloxidase activity has also been detected in insect haemocytes exposed to fungal metabolites (Huxham et al., 1989).

The observations on cytochemistry in this chapter have important implications for parasite survival in the haemocoel. Hymenolepis is recognised as foreign and the immune system responds to encapsulate and melanise (phenoloxidase), kill (peroxidase) and degrade (acid phosphatase) the invader. The finding that β1,3-glucans do not affect the enzyme characteristics of the blood cells might imply that this response requires a more intense or varied immunogenic stimulus. Of particular interest is that the enzyme content of the blood cells does not change after infection with Moniliformis. Whether this indicates immune evasion by the parasite is not proved by this experiment but it does shed some doubt on immune suppression as a survival mechanism. Further clarification of the mechanisms responsible for these changes in enzyme content require detailed studies using separated cell populations.

There are many interesting questions arising from this work which need to be answered by more detailed biochemical examination of the haemocytic "peroxidase". Identification of the enzymes involved in capsular melanisation would be an important line of research. Also, by understanding the interaction of the phenoloxidases (tyrosinases and laccases) with "peroxidase", we might be able to target areas to exploit for pest control. Indeed, we might gain some insight into how some haemocoelic parasites avoid melanisation.

SUMMARY

Immunohistochemical and cytochemical methods have been used in this chapter to examine the enzyme content of the cockroach immune system and to relate this to immunocompetence.

1. Acid phosphatase is present in intracellular granules of 90% of plasmatocytes whereas only 30% of plasmatocytes contain peroxidase-positive granules and 40% contain lysozyme-positive granules. In addition, the phagocytic cell subpopulation does not contain peroxidase and this information indicates that the plasmatocyte subpopulation is heterogeneous with respect to function in the immune response.

2. Acid phosphatase is released into the phagosome of plasmatocytes and lysosomal enzymes are therefore likely to be involved in intracellular digestion.

3. Plasmatocytes do not liberate superoxide anion after phagocytosis indicating that a respiratory burst does not occur in these cells. In view of this evidence, the role of peroxidase in the immune response and the possibility that the peroxidase-like activity is due to phenoloxidase is discussed.

4. Hymenolepis has a profound effect on the immune system since infected cockroaches contained relatively far more peroxidase than control animals suggesting that immunocompetence is enhanced in response to infection.

Zs reduced the cellular peroxidase but did not affect the acid phosphatase. Possible explanations for the differences between Zs and

Hymenolepis are discussed.

5. Acanthellae of Moniliformis caused a reduction in the proportion of peroxidase-positive cells which could represent diminished immunocompetence caused by immune suppression.

CHAPTER 7 - GENERAL DISCUSSION

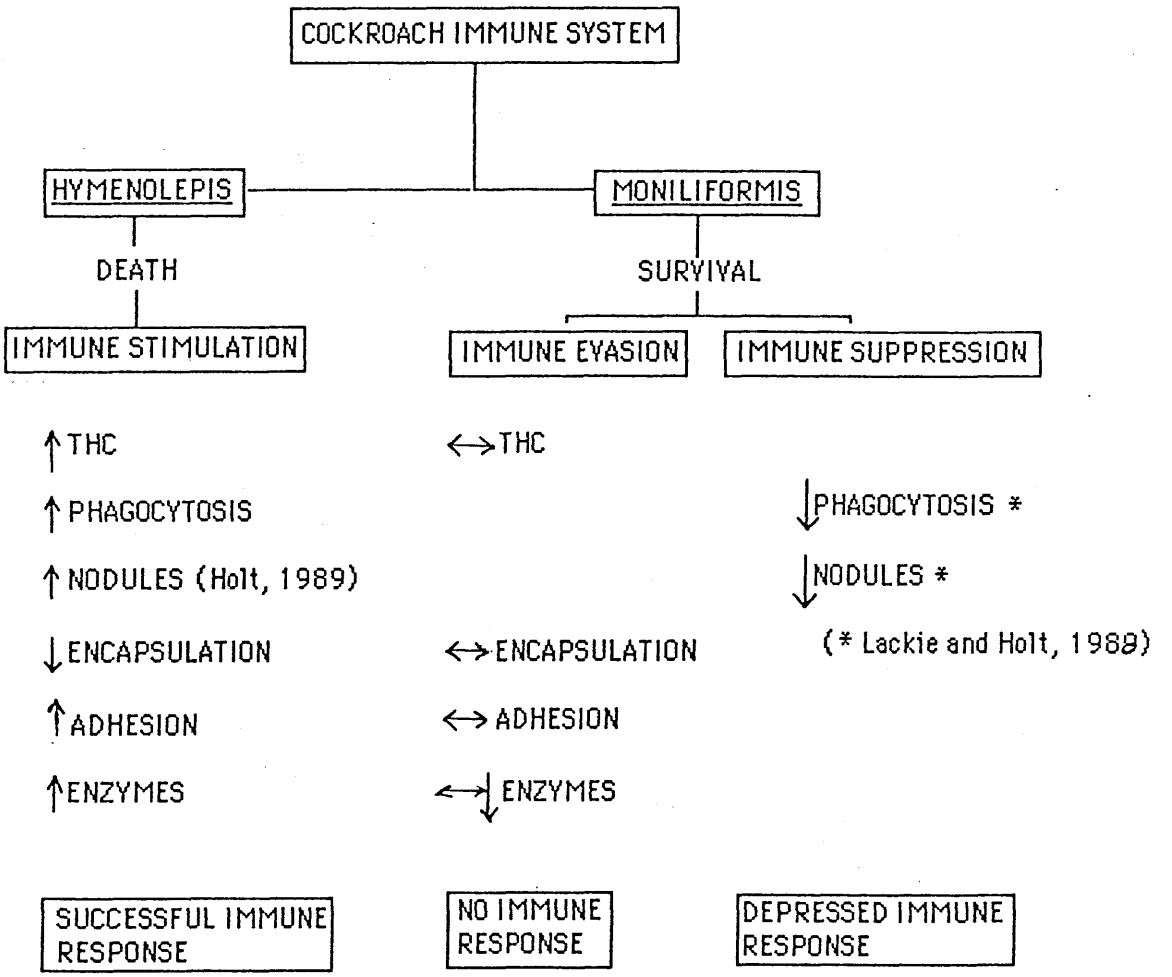


FIGURE 7.1
EVIDENCE FOR IMMUNE ACTIVATION BY HYMENOLEPIS AND IMMUNE EVASION AND SUPPRESSION BY MONILIFORMIS

CHAPTER 7 - GENERAL DISCUSSION

A discussion section and summary is included at the end of each chapter to review the findings of individual chapters. To summarise and discuss the overall results of the work, this general discussion has been included. The emphasis of the discussion will be evaluation of the results from the viewpoint of host resistance and susceptibility to helminthic infection and to discuss a hypothesis for parasite survival in insects. Figure 7.1 summarises the evidence for immune activation by Hymenolepis and either evasion or suppression of host cellular immunity by Moniliformis larvae, derived from the present work and that of other authors.

The overall aim of the project was to clarify the cellular responses of P. americana to H. diminuta and M. moniliformis. Haemocyte behaviour was assayed to estimate the discriminatory abilities of the immune system and the competence to respond to infection. The assays used to test immunocompetence were the circulating cell number, phagocytic capability of haemocytes, nodule formation (Chapter 3), encapsulation in vivo and in vitro (Chapter 4), and blood cell cytochemistry (Chapter 6). The acuity of host non-self recognition was examined by assessing haemocytic adhesion to specific non-self molecules (Chapter 5).

In the following section, the hypothesis that the immune system of the cockroach is stimulated by and can respond to non-self molecules (Zymosan A supernatant and Hymenolepis) is discussed. Later sections will deal with the evidence for immune suppression or evasion as survival strategies for Moniliformis larvae. Possible strategies for immune evasion or suppression by parasites has been thoroughly reviewed (Lackie, 1986b; Yoshino and Boswell, 1986; Christensen and Tracy, 1989)

and the reader is referred to these texts for detailed discussion of invertebrate parasite-host interactions.

7.1. IMMUNE ACTIVATION

Hymenolepis

The overall question to be addressed in this section is how does the cellular immune system of a resistant host respond during infection?

When Hymenolepis larvae are injected into cockroaches, the immune system reacts and they are encapsulated by host haemocytes. In the present work, several behavioural changes have been observed in host haemocytes which imply parasite-induced immune activation. Hymenolepis oncospheres increased the cell number in infected individuals relative to controls (Chapter 3). However, an important consideration in this context is whether these cells are mature, competent cells. The observations on cell spreading are important since this property provides clues on the capacity to form effective capsules and as well as the extent of differentiation of invertebrate blood cells. The cells exhibited no changes in spreading and phagocytic uptake was greater implying that the elevated cell count in Hymenolepis-infected cockroaches is composed of competent cells. Sminia and Barendsen (1980) working on snail blood cells showed that small round haemocytes had a low intracellular enzyme content and phagocytic capacity but that these same cells differentiated into large, phagocytic, spreading cells with a high enzyme content. It is not known whether the lysosomal enzymes play a direct role in the death of Hymenolepis oncospheres injected directly into the haemocoel (Holt, 1989a) but haemocyte subpopulations, identified on the basis of peroxidase staining, exhibited differences

in cytochemical staining characteristics after infection (Chapter 6); a larger proportion of haemocytes contained more enzyme. Whether this represents a qualitative change in the subpopulations or a quantitative change in the enzyme only is unclear. Nonetheless, the overall amount of this enzyme in the immune system is increased since the total cell count is also higher after infection.

The evidence discussed above demonstrates quite clearly that the cellular immune system of the cockroach responds to infection with Hymenolepis. However, for an immune reaction to occur, the foreign object must first be recognised as "non-self". Many authors have transplanted allogeneic (ie. intraspecific) and xenogeneic (ie. interspecific) tissues to insects to investigate the ability of hosts to recognise and respond to foreign tissue (Salt, 1970; Lackie, 1983, 1976; George, Karp and Rheins, 1984). With the development of immunological and molecular biology techniques, lectins have emerged as likely recognition factors in invertebrates. The results on cell adhesion and parasitism (Chapter 5) provide persuasive evidence for membrane-bound lectin on haemocytes being involved in defence against Hymenolepis. If haemocyte membrane lectin expression is enhanced during infection, it is possible that the sugar moities on the cells are also altered to improve cell-cell adhesion (nodule formation) or cell co-operation. Nappi and Silvers (1984) showed that infection of Drosophila larvae with parasitoid wasps was associated with a change in the proportion of haemocytes which stained with the lectin, wheat germ agglutinin (WGA). A high percentage of circulating WGA-stained cells was detected in insects successfully encapsulating parasitoid eggs and these authors suggested that immune competence was due to alterations in the cell surface which augment cell-cell and cell-substratum

adhesion.

Further work using this system points to exciting prospects for enhancing our general knowledge of the parasite-host interaction and, more specifically, the molecular basis of "non-self" recognition in insects.

In summary, it seems that Hymenolepis in the gut has a marked effect on the immune system of the cockroach host, as indicated by several behavioural assays.

Zymosan A Supernatant

Relative to the Hymenolepis infection, Zs seems to have less impact on several aspects of the cockroach immune response. In the present study, infection with the parasite caused a change in phagocytic uptake, specific adhesivity and enzyme content of the cells whereas Zs did not. Also, Zs reduced the cell count which was the opposite to the parasite situation. The differences between Zs and Hymenolepis in their effect on the cellular immune system of the cockroach are difficult to resolve satisfactorily although some possible explanations can be given.

According to the evidence it seems that P. americana can discriminate and respond differently to different stimuli and it is possible that the recognition and activation mechanisms of the haemocytes for B1,3-glucans and Hymenolepis are not the same. In the absence of particulate material, Zs injection may elicit an exaggerated wound response whereas for Hymenolepis activation the cockroach responds as to a chronic infection and invests energy in the production of toxic enzymes. Nonetheless, Takle (1986) found that Zs was capable of activating components of the prophenoloxidase pathway in cockroach haemocytes, and

Zs has been shown to induce nodule formation in cockroaches (Gunnarsson and Lackie, 1985; present study). Clearly then, Zs does have some actions on the blood cells. However, compared with Zs, Hymenolepis is potentially both a stronger stimulant, because of the diversity of surface antigens on living material, and is longer-lived since nodule formation due to parasite stimulation can be detected in cockroaches up to five days after feeding (Holt, 1988).

In summary, the results suggest that both Hymenolepis and Zs activate cellular immunity in the cockroach but that the mechanism for this stimulation may be different.

7.2. THE NATURE OF THE STIMULUS

That Hymenolepis stimulates the effector mechanisms of the immune system of P. americana is clear from the results presented in this thesis. However, the means by which the parasite induces behavioural changes in the haemocytes even from within the gut is less clear. Interestingly, acanthors of Moniliformis also stimulated the immune system during the initial stages of penetration and establishment within the haemocoel (Chapter 3). Since immune stimulation is not characteristic of established infections of Moniliformis, the stimulus which initiates the immune response is clearly only present during the early developmental stages of the parasite. Direct contact of the immune cells with the parasite tegument might explain immune activation by early stages of Moniliformis if the mechanism for survival by these parasites is not immediately in operation on arrival in the haemocoel. However, oncospheres of Hymenolepis seem to be incapable of successfully penetrating the very muscular gut wall of cockroaches (Holt, 1989a) so that direct contact with the haemocytes is unlikely to

explain immune activation by this parasite.

Whether successful penetration and parasite survival in the haemocoel ensue (Moniliformis) or not (Hymenolepis) damage to the gut tissue seems inevitable as the rostellar blades of the acanthor and the hooks of the oncosphere tear at the tissue to effect entry. Wound factors produced by damaged tissues could contribute to the immunostimulatory effect of Hymenolepis and the early stages of Moniliformis. Unfortunately, studies of the Hymenolepis-infected cockroach guts failed to provide evidence for damage associated with Hymenolepis infection, even though the parasite does hatch successfully within the cockroach gut (Holt, 1989a).

Wounding of the gut tissue may explain the initial changes in the THC after infection since cuticular wounding also alters the circulating haemogram (Guzo and Stolz, 1987; Dunphy and Webster, 1985; Gunnarsson, 1987). Although wound healing may be the initiator for the anti-parasite immune response (Lackie, 1986), eg. the encapsulation of Moniliformis acanthors as they penetrate the gut, other factors are likely to be involved. Brennan and Cheng (1975) suggested that the initial stimulus for haemocytes to adhere to Moniliformis acanthors was surface damage to the parasites themselves during penetration. The results of Yamamoto and co-workers (1985) and confirmed by Christensen and Tracy (1989), would also appear to argue against gut damage as being the only mechanism for immune activation. These authors have reported that in the mosquito, Armigeres subalbatus the response to Brugia malayi and B. pahangi is highly species specific even though both parasites are morphologically very similar and both infect the host orally.

An alternative hypothesis to wound factor-induced immunity might be that soluble molecules derived from the parasite, possibly during parasite hatching, pass across the gut to act on the blood cells. In a series of papers, Rogers (1958, 1960, 1961) found that hatching of Ascaris lumbricoides embryos is facilitated by the production of a chitinase, a lipase and possibly, a protease. Chitinase produced by a hatching parasite could also weaken the chitinous peritrophic membrane of the host midgut (Whitfield, 1971) to help parasite penetration. It is plausible then that enzymes produced to aid hatching could pass across the gut wall and alert the immune system to the parasites in the gut. Interestingly, Glinski and Jarosz (1986) induced the internal defence response of larvae of the wax moth, Galleria mellonella, by feeding with the bacterium, Serratia marcescens, but these authors did not propose how the bacteria in the gut might stimulate the host immune system. More recently, Gunnarsson (1988) has reported that haemocytes aggregate around the cuticular penetration sites of the fungal pathogen, Metarizium anisopliae, even before the hyphae have penetrated the cuticle. This activation without direct contact with immune effectors deserves more attention since, by non-specifically enhancing the competence of the immune system, the host may become more resistant to infection.

7.3. MONILIFORMIS - IMMUNE SUPPRESSION OR EVASION?

Immune Suppression - The Evidence

Immune suppression by a parasite is a dangerous strategy since the host becomes less able to deal with a subsequent infection which could then lead to the premature death of the host. Several authors have reported

suppression of the immune responses in parasitised hosts, particularly by parasitoid wasps (Streams and Greenberg, 1969; Rizki and Rizki, 1984; Davies and Vinson, 1986; Stolz and Guzo, 1986). Immune suppression has also been reported to occur in locust hosts (Schistocerca gregaria) infected with the nematode, Mermis nigrescens (Ibrahim et al., 1986) and in mosquitoes infected with the filarial worm, Brugia pahangi (Christensen and LaFond, 1986). Bitkowska et al. (1982) have also presented preliminary evidence for suppression of host encapsulation and recognition by Trypanosoma cruzi within the gut of the bug, Triatoma infestans. Also, Echinostoma paraensei sporocysts are thought to interfere with the cellular immune system of the snail host (Loker et al., 1986). Fungal metabolites, called destruxins, which are produced by the entomopathogenic fungus, Metarizium anisopliae, have a suppressive effect on the nodule-forming ability (Huxham et al., 1986; Vey et al., 1988) and phenoloxidase activity (Huxham et al., 1989) of insect haemocytes.

Previous authors have suggested that Moniliformis has an immunosuppressive action on host cockroaches and this permits the worm larvae to survive in direct contact with the immune effectors (Lackie and Holt, 1988; Brennan and Cheng, 1975). The THC data from acanthellae-infected cockroaches do not support this hypothesis as a suppressed immune system may be expected to contain fewer circulating cells. It seems reasonable to conclude then that Moniliformis could inhibit the competence of the immune system by affecting the functioning of the cells during infection. However, plasmatocytes from Moniliformis infected animals appear, at least morphologically, to retain the ability to phagocytose, spread and adhere to each other to form nodules or capsules around Sepharose beads.

Immune suppression and evasion need not be mutually exclusive and a combination of both would provide the parasite with several safety nets in the event of recognition and attack by the host. In this way, selective inhibition of cellular responses to challenge (eg. phagocytosis, nodule formation, melanisation (Lackie and Holt, 1988)), while leaving other responses intact (eg. THC, enzyme content, encapsulation ability and adhesion), could permit survival of the parasite in the host while retaining the capacity to respond to infections with opportunistic organisms. Rizki et al. (1990) have similarly reported that Drosophila haemocytes, while incapable of encapsulating parasitoid eggs, are still able to form melanised nodules and encapsulate dead supernumerary parasites.

A major drawback to the immune suppression hypothesis as being the only survival mechanism for Moniliformis larvae relates to the length of time for the effect to become manifest. Parasites raised in one cockroach host then transferred to another would be attacked by the immune system of the second naive host, unless the suppressive effect is localised (Brennan and Cheng, 1975) or occurs on direct haemocyte contact with the parasite. Since larvae experimentally transferred to naive hosts are not encapsulated and survive well, widespread systemic suppression of the host would have to be instantaneous. Brennan and Cheng (1975) also held this view of a localised inhibition of cellular functioning rather than a widespread depression in the immune system. These authors suggested that tyrosinase activity was reduced at the surface of the larvae so preventing formation of toxic quinones and melanin. However, their technique of injecting whole homogenised larvae into cockroaches is likely to have induced major, widespread effects on

the immune system in a generalised response to foreign material rather than to a defined fraction of the homogenate.

Immune Evasion - The Evidence

Compared with immune suppression, immune evasion is probably more difficult to evolve since the parasite would be susceptible to the host defences until it resembled host tissue sufficiently to be overlooked by the host immune system. Also, by adapting to resemble one host, the parasite is likely to effectively restrict itself to survival within only that host. However, once adapted to its' host, immune evasion is probably less energy-requiring to maintain than suppression and therefore, is more likely to occur in long-lived parasites which require host fitness.

The evidence presented in this thesis points to immune evasion as the method by which Moniliformis larvae escape attack. If we postulate that Moniliformis evades the recognition arm of the immune system then we expect the cells to be no more or less responsive to other particles or molecules. In the lectin-ligand cell adhesion studies (Chapter 5), the cells from acanthellae-infected animals showed no changes in adhesion to the glycoproteins. Therefore, if the haemocyte lectins are involved in cellular recognition (see Chapter 5 for discussion), then the results imply that Moniliformis is not recognised as foreign in the same way as Hymenolepis. However, recognition of "non-self" is likely to be a complex process. It is worth considering here whether Moniliformis could differentially inactivate haemocyte surface recognition sites so that all the cells fail to recognise the parasites as foreign but can still distinguish the glycoproteins. Alternatively, if haemocytic function is compartmentalised, with different

subpopulations co-operating in immune processes, then inactivation of the "helminth-specific" subpopulation could allow the parasite to escape an otherwise competent recognition system.

However, as discussed above, immune suppression and evasion need not be mutually exclusive and the parasite might use both mechanisms to survive. In addition to the work in this thesis, other evidence for immune evasion by this parasite exists in the literature.

Moniliformis larvae may acquire host antigen to survive in the host haemocoel. O'Brien et al. (1986) have found that the parasite envelope contains identical proteins to host serum. These authors have also reported the presence of connective tissue-like macromolecules, called glycosaminoglycans, using cytochemical staining of the parasite envelope. However, it is not known whether these molecules are host-derived and adsorbed onto the parasite envelope or encoded in the parasite genome. The possibility that the host-like molecules on the envelope are involved in immune evasion, perhaps by masking foreign parasite antigens, is appealing.

Further evidence for immune evasion rather than suppression, has been provided by Lackie (1972b). Working on the assumption that parasite survival would be enhanced in immune suppressed animals, he found that subsequent infections of Moniliformis in already-parasitised animals were no more successful than initial infections.

To investigate the mechanism of parasite survival and host specificity, Lackie and Lackie (1979) reared Moniliformis larvae in locusts then experimentally transferred the larvae to cockroaches. If the parasites expressed locust antigens then, using this experimental procedure, they could be highly vulnerable to recognition and attack by

the cockroach immune system. However, these larvae were not affected by the cockroach immune cells, perhaps indicating that they do not adopt host antigens to evade host immunity.

Molecular mimicry, in which the host-like determinants are encoded in the parasite genome, is another method used by parasites to avoid recognition. However, this mechanism is unlikely to explain why larvae transplanted into cockroaches from locusts avoided attack since the parasite would need time to manufacture and express cockroach-like surface molecules. With the molecular parasitology techniques presently available, solutions to these problems are now much more accessible than previously.

The experiments of Lackie and Holt (1988) suggested immune suppression of the cockroach host by Moniliformis larvae. However, in contrast with most of the work presented here, these authors examined the immune response of parasitised animals to challenge. A model which incorporates both immune evasion and suppression can be proposed such that animals parasitised with Moniliformis retain an effective immune system. From the present work, it seems that the developing Moniliformis larvae do not suppress the effector mechanisms of the unchallenged host. However, on challenge, the usual cellular response is dampened by the suppressive effects of the parasite (Lackie and Holt, 1988). Thus, the competence of the host immune system is adequate to avoid a potentially fatal infection but limited by the parasite larvae. In this way this parasite, which requires a relatively long period for development, ensures the survival of its host while avoiding recognition and immune attack.

CONCLUDING COMMENTS

From the above discussion it is apparent that many fundamental questions on host immunity and resistance to infection remain to be answered using this model system. Further studies are required to clarify the nature of the stimulus by which Hymenolepis activates the immune system of the cockroach, apparently without any direct contact with the blood cells. The means by which Moniliformis larvae circumvent the host immune response awaits further research since the present work favours immune evasion whereas previous authors have provided evidence for immune suppression.

Of particular interest to myself and following on from the present study, would be further work on the role of membrane-bound lectins in immune recognition and cell sub-populations after stimulation. Finally, the question of killing and the "peroxidase or phenoloxidase" problem arising from Chapter 6 desperately needs attention since, ultimately, killing of the parasite is the aim of the host, if not the researcher.

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APPENDIX

1. SOLUTIONS

Cockroach Maintenance Medium - D_{73}

Amino acids	mg/200ml
L-arginine hydrochloride	60
L-aspartic acid	50
L-cysteine	52
L-glutamic acid	190
glycine	130
L-histidine hydrochloride	52
L-isoleucine	48
L-leucine	50
L-methionine	100
L-phenylalanine	40
L-proline	134
L-serine	16
L-threonine	40
L-tryptophan	40
L-tyrosine	20
L-valine	30

The amino acids were obtained from Sigma Chemical Co., Poole, unless otherwise stated.

Salts	mg/200ml
Nacl	1200
KCl	160
$MgSO_4(7H_2O)$	36
Na_2HPO_4	10
KH_2PO_4	12
$CaCl_2(2H_2O)$	28

Other Additions	mg/200ml
D-glucose	200
Hepes	952
Gentamycin (sulphate)	40
Streptomycin (sulphate)	20
Penicillin (sodium salt)	0.3
Cynocobalamine	$1x10^{-3}$
Flow BME Vit. (x100 soln.)	1ml
Flow Medium 199 (x10 soln.)	8ml

The above was made up to 200ml with double distilled water and the pH adjusted to 7.2-7.3. The final solution was filter sterilised with a 0.22um Millipore filter. All ingredients are Sigma grade. Modified from D73 medium of Quiot et al. (1985), In Vitro 21: 603.

CitrateEDTA (calcium/magnesium free) Anticoagulant

	g/l	mM
Tri-sodium citrate	8.82	32.0
Citric acid	5.46	26.0
NaCl	5.30	90.7
D-glucose	18.00	100.0
EDTA	3.72	10.0

The above was made up to 1L with double distilled water and adjusted to pH = 4.6 with 5M NaOH, osmolarity = 350mOsmol. The final solution was autoclaved to sterilise.

Hepes Buffered Saline (HBS)

	g/l	mM
NaCl	7.60	130.0
KCl	0.74	10.0
$MgCl_2(6H_2O)$	0.41	2.1
$CaCl_2(6H_2O)$	0.44	2.0
Hepes	2.38	10.0

The above was made up to 1L with double distilled water and adjusted to pH = 7.2 with 5M HCL. The final solution was autoclaved to sterilise.