ONTOGENETIC STUDIES ON THE IMMUNE SYSTEM OF TWO LOCUST SPECIES: *LOCUSTA MIGRATORIA* AND *SCHISTOCERCA GREGARIA*

by

AMIRA ALY ABU EL-MAGD TOLBA

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SUMMARY

Work presented in this thesis examines the ontogenesis of immunity in two species of locust, Locusta migratoria (the migratory locust), and Schistocerca gregaria (the desert locust). Three haemocyte types (granulocytes, coagulocytes and plasmatocytes) were observed in all stages examined and the differential blood cell count remained constant throughout development. Following antigenic challenge in all developmental stages, there was an increase in the percentage of blood granulocytes, a decline in coagulocytes and less consistent changes were noted in the percentage of plasmatocytes. Autoradiographical studies revealed proliferative activity in haemopoietic tissue following antigenic challenge but not in peripheral haemocytes. Inducible agglutinins were observed in the haemolymph following challenge with vertebrate erythrocytes and bacteria. Agglutinin titres varied with species, developmental stage and the antigen used. Opsonisation studies revealed that haemolymph from immunised Locusta could promote phagocytosis by haemocytes in vitro.

Haemocytes of both species were able to phagocytose vertebrate erythrocytes, charcoal and indian ink particles in vivo. The phagocytic response to erythrocytes was stronger than that observed towards inert particles and phagocytic responses were weaker or absent in younger stages. Adult and fifth instar haemopoietic tissue was active in phagocytosis of charcoal and indian ink, but that of younger stages failed to trap foreign particles.

Haemocytes of adult and fifth instar nymphs were seen to respond positively in chemotaxis and chemokinesis assays. Positive responses were observed following exposure to erythrocytes and bacteria, and haemocyte capsules formed around cat gut implants also produced chemotaxis and chemokinesis responses. Schistocerca gregaria failed to encapsulate implants and, in general, responses in this species were weaker than those observed in Locusta migratoria.

Key words:
Ontogeny of insect immunity.
Haemocytes of Locusta migratoria and Schistocerca gregaria.
Phagocytosis, encapsulation and agglutinins in locusts.
Chemotaxis and chemokinesis in locust haemocytes.

To the memory of my dear brother Hany

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CHAPTER I

GENERAL INTRODUCTION

The immune systems of invertebrates have attracted increasing attention in recent years (Cooper, 1974; Maramarosch and Schope, 1975; Angus et al., 1976; Manning and Turner, 1976; Solomon, 1980; Ratcliffe and Rowley, 1979b, 1981; Lackie, 1980, 1986; Cheng, 1984; Ratcliffe et al., 1985; Brehelin, 1986). The reasons for this renewed interest in invertebrate defence mechanisms are varied but three recurrent themes have been emphasised by Brehelin (1986).

The first concerns a fundamental interest in discovering new defence mechanisms in invertebrates, and the use of phylogenetic studies to explain the more complex immunological phenomena of higher vertebrates. In order to survive, all animals have evolved some kind of response to recognise and eliminate non-self without destroying self (Olafsen, 1986). However, there are still many fundamental gaps in our knowledge of invertebrate defence mechanisms. This lack of information results in part from the enormous diversity of invertebrate phyla available for study. Each phylum differs markedly from other phyla in terms of overall body plan and structural complexity and these variations have allowed invertebrates to successfully colonise a wide variety of habitats. This variability is also reflected in the structure and function of invertebrate defence systems and the molecular basis for discrimination between self and non-self, a crucial

distinction at all levels of organisation of living matter, remains one of the most intriguing puzzles of immunobiology.

A second reason for studying invertebrate defence mechanisms is to advance understanding of how invertebrates react to parasites or pathogens. This information has important implications for the biological control of agricultural insect pests. In order to control such pests it is necessary to select for pathogenic strains of parasite which can evade or overcome the immune response of the host and kill it. Many invertebrates (eg. insects and molluscs) also act as intermediate hosts or vectors of disease which place a considerable economic and social burden on man. Lackie (1986) has pointed out that it may be possible to manipulate invertebrate host-parasite relationships by selecting for strains of insect whose immune response can effectively control and kill the parasite. There is clearly a need for a wider understanding of invertebrate defences before such hopes of biological control can be realised.

Finally, another area of applied research involving invertebrates concerns those species with commercial potential. Recent advances in aquaculture have given rise to the intensive farming of crustaceans and molluscs. High density rearing and maintenance techniques clearly provide an ideal environment for the transmission of disease amongst farm stocks, and the development of commercial farming requires a better knowledge of the ways in which crustaceans and molluscs resist infection and how this resistance may be improved by genetic selection or vaccination (Brehelin, 1986).

In general, invertebrate defence mechanisms are less complex than those of vertebrates which exhibit integrated cell-mediated and humoral immunity, memory, complex immunoregulatory mechanisms and multiple molecular classes of immunoglobulins (Hildemann, 1974). Although invertebrates lack some of these features (notably immunoglobulins) they are still normally able to keep their internal body fluids sterile and it seems beyond doubt that they have evolved adequate means to distinguish between self and non-self (Bang, 1973). Figure 1.1 provides a summary of the immunopotentialities of different invertebrate phyla compared with vertebrates.

1.1. HAEMOCYTES AND IMMUNITY IN THE INVERTEBRATES: A BRIEF OVERVIEW

The existence of immunological reactivity at the various levels of invertebrate phylogeny is now well established, and a summary of immunological reactivities in invertebrate phyla is outlined in Fig.1. Transplantation experiments have shown that invertebrates have the ability to recognise 'self' from 'non-self' (Burnet, 1974; Cooper, 1986; Cooper and Roch, 1984; Lackie, 1983a, 1985b, 1986a). Immunoglobulin-like molecules in invertebrate body fluids are absent in all species examined (Whitcomb et al. 1974), so that recognition of foreignness may be by serum independent mechanisms, or else by other types of soluble recognition factors present in invertebrate fluids. In the search for such recognition factors the naturally occurring agglutinins found in the body fluids of many invertebrates have been considered to act like a primitive type of antibody molecule and have been shown to combine with natural antigens such as sugars on bacterial

and erythrocyte surfaces (Acton and Weinheimer, 1974). Tripp (1966) was the first to show that haemagglutinins played an opsonic role in the uptake of erythrocytes by insect haemocytes. Further reports (Stuart, 1968; Prowse and Tait, 1969; McKay and Jenkin, 1970; Pauley et al., 1971a; Tyson and Jenkin, 1973, 1974; Paterson and Stewart, 1974; Anderson and Good, 1976) on various molluscs and crustaceans, have shown similar results with both bacterial agglutinins and haemagglutinins present in the haemolymph which acted as opsonins.

One of the greatest problems facing comparative immunologists is the existence of inconsistent nomenclature in the literature for the blood cell types of invertebrates. Ratcliffe and Rowley (1979b) have summarised the structure and function of the blood cells of different invertebrate groups, and a brief survey is presented below.

As may be seen from Fig.1, far less is known about the immune capabilities of the acoelomate phyla, although 'cell mediated immunity' has been demonstrated in both sponges and corals by Hildemann and his co-workers (Hildemann et al., 1977; Hildemann and Benedict, 1975). The reactions described show primordial cell mediated cytotoxic reactions, together with alloimmune memory.

1.1.1. Protostome immunity

1.1.1.1. Crustaceans:

These arthropods have an open circulatory system except in the higher forms which have a partially closed system. The haemocytes of arthropods are mesenchyme cells but in some crustacean species they

originate from haemopoietic organs located in the wall of the gizzard called lymphocytogenic nodules (Ghiretti-Magaldi et al., 1977).

Crustacean blood cells appear to fall into two diverse groups; one containing highly refractile granular cells, and the other, hyaline/semihyaline amoeboid cells. The granular cells are often sub-divided according to the numbers, size and staining reactions of the granules while different types of hyaline cells are distinguished on the basis of the nuclear/cytoplasmic ratio (Cornick and Stewart, 1978; Bauchau, 1981; Soderhall and Smith, 1983; Smith and Soderhall, 1983, 1986).

The haemocytes of the Crustacea seem to be actively involved in host defence mechanisms. The hyaline amoeboid cells appear to be chiefly involved in phagocytosis, although in a few species the granular cells are also phagocytic (Wood and Visentin, 1967; Paterson and Stewart, 1974; Ratcliffe et al., 1982; Chorney and Cheng, 1980). Hyaline cells have also been reported to bring about plasma coagulation (Wood and Visentin, 1967). The function of the granular cells in defence reations is unclear but they are often highly fragile and they may act in haemolymph coagulation and/or the release of agglutinins (Wood and Visentin, 1967).

1.1.1.2. Annelids:

The annelid worms are characterised by a coelom and a closed blood vascular system. Localised haemopoietic organs have rarely been found in the annelids (Stephenson, 1930; and Dales, 1967), but it is believed that the coelomic cell aggregates in the anterior gut and the nephridial

blood vessels may function to produce the free cells in the coelom (Hostetter and Cooper, 1974).

There are two main groups of cells which are clearly distinguishable morphologically and functionally, the phagocytic amoebocytes and the eleocytes. The phagocytic amoebocytes have a graded development: from immature stem cells of limited phagocytic ability similar to insect prohaemocytes, to larger granule-containing cells which are very actively phagocytic. These mature cells are usually subdivided according to the staining properties of their cytoplasmic granules into basophils, neutrophils and acidophils (Cameron, 1932; Hostetter and Cooper, 1974; and Stein et al., 1977). Annelid phagocytic amoebocytes also form capsules and brown bodies in response to large foreign bodies and bacteria in the coelom (Cameron, 1932), and rapidly aggregate to form plugs as a result of wounding (Stang Voss, 1974).

The second group of annelid coelomocytes includes the eleocytes which may be derived from the chloragogen cells surrounding the digestive tract (Ratcliffe and Rowley, 1979b). They may also originate by transformation of amoebocytes (Dales, 1964). The main function of the eleocytes is probably in the nutrition of the developing gametes (Liebmann, 1946).

Cooper (1965a, 1965b, 1968, 1969a, 1969b, 1973) pioneered experiments on transplantation in annelids and his studies have stimulated many people to make further attempts to see if anamnestic responses do occur among the invertebrates. These are reviewed by Cooper (1980, 1986) and Cooper and Roch (1984) and have demonstrated

specific skin transplantation immunity and memory in annelids. Annelids reject both xenografts and allografts. The speed of rejection is directly related to temperature. Increasing the reaction temperature tips the balance toward positive rather than negative memory. The rejection process involves invasion of the graft by cells (Coelomocytes), and these can transfer antixenograft immunity to a non-immune worm. In addition, increased numbers of coelomocytes are associated with second-set grafts compared with first-set grafts, thus providing a cellular basis for the gross manifestations of memory (Chateaureynaud-Duprat and Izoard, 1973, 1977; and Chateaureynaud-Duprat and Lestage, 1980). In addition to graft rejection, earthworm leucoytes have also been described as reacting in mixed leucocyte reactions in vitro (Valembois et al., 1981). Furthermore, Roch and co-workers have shown that earthworm leucocytes respond to mitogens (Phytohaemoagglutinin, Concanvalin A and Lipopolysaccharide) by increased DNA synthesis. As with vertebrates, separate cell populations respond to different classes of mitogen (Roche and Valembois, 1977).

In addition to cell-mediated immune reactions, annelids also possess a range of soluble factors with a number of described activities. These have recently been reviewed by Valembois et al. (1986) and only the major points are mentioned here. Earthworms produce both haemolysins and haemagglutinins. Du Pasquier and Duprat (1968) were able to demonstrate that the haemolytic factor present in Eisenia foetida andrei was capable of lysing erythrocytes of frog, sheep and human origin. Furthermore, by using a modified Jerne's plaque assay they were able to show that a haemolytic factor was released by the chloragocytes. Haemagglutins have also been described in the coelomic

fluid of annelids against a range of vertebrate erythrocytes including sheep, human, rabbit, rat, badger, chicken and frog (Valembois et al., 1986). Chloragocytes are also thought to be involved in the synthesis of haemagglutinins. Antibacterial molecules with bacteriostatic and bacteriolytic properties have also been described in annelids including polychaetes and hirudinea. Some of this lytic activity has been ascribed to lysozyme but other molecules are also thought to be involved. Many of the soluble factors present in annelids are inducible, but lack the fine specificity of vertebrate immunoglobulins. They probably recognise a range of surface molecules (carbohydrates?) present on the surface of a wide variety of foreign cell surfaces.

1.1.1.3. Molluscs:

The molluscs exhibit tremendous variability, and range from relatively simple and even parasitic forms to highly developed ones such as the squid and the octopus (Bang, 1975). Molluscs usually have a haemocoele with an open circulatory system, a heart, venous sinuses and arteries (Hill and Welsh, 1966). Little is known about the origin of molluscan haemocytes (Malek and Cheng, 1974) and there are no well-defined haemopoietic organs, but various parts of the body, such as the mantle epithelium and connective tissue, walls of blood sinuses, connective tissue around the kidney and the digestive gland can give rise to blood cells (Sminia, 1974; Sminia and Barendsen, 1980).

The blood cells of molluscs fall into two categories which, like those of the crustacea, contain granular cells and hyaline and semi-hyaline cells. The haemocytes of molluscs are involved in excretion, digestion, nutrition and shell repair and host defence reactions. The

latter reactions include phagocytosis, encapsulation and wound healing (Betances, 1922).

A number of authors all report that molluscs readily phagocytose injected particles including erythrocytes, bacteria, bacterial spores and yeast (Sminia and Van Der Knaap, 1986); Sminia et al., 1979, 1981; and Tripp (1970). Molluscs also encapsulate many kinds of foreign materials in their tissues such as eggs and larvae of parasites, pollen, certain bacteria and implanted tissues.

Work on transplantation reactions in molluscs has suffered from technical difficulties, and little decisive evidence is available. The animals were often unable to withstand repeat grafting and prolonged observation at elevated temperatures.

Natural haemagglutinins with varying degrees of specificity for diverse foreign erythrocytes have been described in the body fluids of several molluscan species. Serum factors in molluscs have been recently reviewed by Sminia and van der Knaap (1986).

In contrast to the protostome groups described above, the deuterostomes have received rather less attention from comparative immunologists. This is perhaps surprising when it is considered that the vertebrates sprang from this group. Nevertheless, a number of studies have been carried out and these are briefly reviewed below.

1.1.1.4. Echinoderms:

These are exclusively marine, coelomate and have a blood vascular system which is unique and consists of strands of Pacunar tissue. (Borrodaile et al., 1961). Andrew (1965) studied the origin of the coelomocytes in the echinoderms, and suggested that leukopoietic elements occurred in the oesophagus, whilst mesenchyme elements were to be observed in the walls of the blood vessels and in the ovoid glands. Wright (1976), however, reported that such localised blood-forming tissues did not exist and that coelomocytes were released first from the coelomic epithelium and then divided in the circulation.

The blood cell picture in the echinoderms is quite complicated. Boolootian (1962) identified 14 different cell types, but Smith (1981) simplified these into four morphologically distinct types. These include phagocytic leucocytes (amoebocytes), vibratile cells, red spherule cells and colourless spherule cells. They are involved in immunological reactions such as phagocytosis, clotting or cell aggregation, cell clumping (which resembles nodule formation in insects) and also encapsulation of invaders.

Phagocytosis by echinoderms was first studied by ~~Durham (1888) and~~ Metchnikov (1892) followed by Metalnikov (1927). Phagocytosis of bacteria in the haemocoele of sea stars has been shown (Bang and Lemma, 1962; Johnson, 1969; and Smith, 1981) and the phagocytic cells of echinoderms may also function during clotting or cell aggregation (Johnson, 1969). Boolootian and Giese (1958 and 1959) believed that true extracellular coagulation occurs and is initiated by the hyaline haemocytes. Cell clumping also takes place in response to bacterial

invasion and the component cells have been reported to secrete a sticky substance (Bookhout and Greenburg, 1940; and Bang and Lemma, 1962). Chien et al. (1970) also mentioned that echinoderms could encapsulate and isolate invaders.

Hildemann and Dix (1972) demonstrated some years ago that, under optimal conditions, echinoderms showed unequivocal evidence of specific allograft immunity with at least short term memory. More recently, Brillouet et al. (1981) were able to demonstrate that starfish leucocytes proliferate in response to different mitogens. Furthermore, they were able to show that mitogen-stimulated cells produced a "lymphokine-like" factor which enhanced division in cultured starfish cell populations. After separating starfish leucocytes on the basis of their adherence to nylon wool columns, they discovered a non-adherent population which responded to mammalian T-cell mitogens and an adherent population which responded to T-independent mitogens. These properties are of considerable interest in considering the key place of the echinoderms in the evolution of vertebrate lymphoid tissue and the phylogenens of the immune response.

1.1.1.5. Tunicates:

The tunicates or urochordates are without a discernable coelom, and possess an unusual blood vascular system consisting of a tubular heart, which drives blood first one way and then the other way by reversal of the contractions. The heart has blood vessels at each end which have no true walls but are just spaces between different structures (Borradaile et al., 1961). Blood forming cells originate from mesodermal cells called haemocytoblasts (Cowden, 1968). Haematogenic tissue is found in

the pharyngeal wall, around the digestive tract and/or in the body wall, and it may be diffuse or organised into small "lymph nodes". (Ermak, 1976; and Wright, 1976). The types of blood cells cited are lymphocytes, phagocytes, vacuolated cells or storage cells, but coagulation cells are absent and clotting involves the aggregation of cells (Ratcliffe and Rowley, 1979b).

Immunological reactions in tunicates have been reviewed by Manning and Turner (1976). If foreign erythrocytes are injected into tunicates, they are first agglutinated, but their subsequent fate depends on the dose injected: if few in number, the cells will be phagocytosed, if larger doses are given, they will be encapsulated, then ejected from the body. A secondary injection of erythrocytes 6 days after the first can elicit an encapsulation response for doses which failed to elicit encapsulation after the first injection. There thus appears to be a memory component, although specificity is lacking. Alloimmunity is present in transplantation experiments, autografts are accepted but allografts are rejected. Lymphocytes of the tunicate Ciona will proliferate in response to the mitogen phytohaemagglutinin. Tunicates also produce a range of lectins capable of binding a range of vertebrate erythrocytes. At least two agglutinin peaks have been identified in body fluids from the ascidian Botrylloides leachii (Schluter et al., 1981). Studies by Coombe et al. (1982) showed that agglutinin levels to sheep or chicken erythrocytes were raised following secondary challenge.

1.2. HAEMOCYTES AND IMMUNITY IN INSECTS

Insects have an open circulatory system in which a dorsal heart drives the blood forward into an anterior aorta which opens into the general body cavity. So the blood bathes all the body systems and organs without the need for a vascular system (Wigglesworth, 1942; Ratcliffe and Rowley, 1979a; and Rowley and Ratcliffe, 1981).

The blood of insects is the medium through which all the chemical exchanges between the organs are effected. Thus, hormones are conveyed to target cells, food is carried from the gut, and waste products are transferred to the excretory organs. It also plays an important role in hatching, ecdysis and the expansion of the wings. A further important function of the blood of insects resides in its protective reactions against foreign particles and invaders (Wigglesworth, 1953). Jones (1962) mentioned that there are loose cells in the body cavity of insects called haemocytes or blood cells. Most of these are normally resting on the surfaces of various organs, but some circulate freely in the blood. The origin of these blood cells (haemocytes) has been studied by many authors. Tauber (1936, 1937) and Ratcliffe et al. (1985) proposed that they may arise from the division of cells circulating in the haemocoel. But Arvy (1952, 1953, 1954, 1956) states that they originate from compact haemopoietic organs within which they may multiply rapidly and differentiate. It has also long been thought that tissues within the pericardial septum (Cuenot, 1891) or tissues within the gonads (Wermel, 1938a, b) could produce haemocytes.

There are over seventy names recorded for the types of insect haemocytes (Gupta, 1979). The naming and classification of the different kinds of haemocytes is often rendered difficult by the varying techniques for staining that have been employed (Hrdy, 1956). Different authors have described widely differing numbers of cell types in their work on different types of insects. In order to simplify this situation, Jones (1962) proposed six basic cell types and Price and Ratcliffe (1974); Ratcliffe and Rowley (1979), and Rowley and Ratcliffe (1981) have subsequently described a classification scheme which is basically a simplified version of Jones's review. This scheme is adopted in the present study. An important point to emphasise is that the highly diverse nature of the insects is reflected in the variable morphology of their blood cells so that homologies even between different insect species may be uncertain (Arnold, 1972).

The six types of haemocytes which are generally present in insects are outlined below:

1.2.1. Prohaemocytes

Prohaemocytes are small round or oval cells, 5-13 m in diameter, in which the nucleus accounts for approximately 75% of cell volume. The thin cytoplasmic rim is undifferentiated although in some species it contains granules (Ratcliffe and Price, 1974).

1.2.2. Plasmatocytes

These cells have been referred to as micro and macro nucleocytes/plasmatocytes, amoebocytes, phagocytes, leukocytes, macrophages etc. (Ratcliffe and Rowley, 1979a). They are larger than

the prohaemocytes, (m) round, oval or spindle-shaped in vivo (Arnold, 1974; Price and Ratcliffe, 1974; Rowley, 1977; and Rowley and Ratcliffe, 1981). The nucleus of the plasmatocytes is in the centre of the cell and occupies approximately 40% of the cell volume. The cytoplasm is finely granulated and contains a variable number of granules and sometimes these granules are found in large numbers and obscure the nucleus. In vitro they attach to glass coverslips by the formation of protoplasmic extensions and usually become amoeboid (Price, 1974). But Brehelin and Zachary (1983) mentioned that these cells are stable under in vitro conditions. The plasmatocytes have been shown to be involved in phagocytosis (Wittig, 1965 and Salt, 1970), encapsulation (Salt, 1970), wound repair (Lai-Fook, 1970 and Bohn, 1975) and nodule formation (Ratcliffe and Gagen, 1976b).

1.2.3. Granular Cells

These cells have many distinct, usually round, colourless, acidophilic granules (Holland, 1909; Jones, 1956, 1959; and Rowley and Ratcliffe, 1976a). Generally, granular cells are round to oval, 8-20 m in diameter, with a central nucleus accounting for approximately 50% of the cell volume (Ratcliffe and Rowley, 1979a). They do not spread on surfaces but Price and Ratcliffe (1974) mentioned that occasionally they may form small thin protoplasmic extensions. The known functions of the granular cells include nodule formation (Ratcliffe and Gagen, 1976b); encapsulation (Schmit and Ratcliffe, 1977); and melanin formation (Schmit et al., 1977, and Ratcliffe, 1986).

1.2.4. Spherule Cells

These are round or ovoid haemocytes with many, very large, usually round, colourless or pale yellow inclusions (Holland, 1909; and Rowley and Ratcliffe, 1981). Spherule cells are capable of breaking down *in vitro* into intensely hyaline forms (Gregoire, 1955, and Jones, 1956) and can release material into the plasma but do not induce coagulation of the plasma (Jones, 1956, 1959). It has been suggested that they are involved in silk production (Nittono, 1960) and may give rise to plasmatocytes (Gupta and Sutherland, 1967).

1.2.5. Cystocytes

Also called coagulocytes by some workers - cystocytes are highly unstable haemocytes. They eject materials, granules or droplets, into the plasma and tend to disintegrate *in vitro* (Yeager et al., 1932, and Rowley, 1977a, 1977b). Cystocytes range in size from 9-14 m and are round to ovoid cells with few to many small, round inclusions (Jones, 1962). The nucleus is usually round and cartwheel-like, and surrounded by a thin rim of cytoplasm (Price and Ratcliffe, 1974).

1.2.6. Oenocytoids

These have been termed oenocyte-like cells and crystal cells by some workers (Ratcliffe and Rowley, 1979a). The cells are relatively large, up to 19 m in diameter with an eccentric nucleus occupying about 20-40% of the cell volume. The cytoplasm encloses only a few granules or globules (Price and Ratcliffe, 1974). They may have a role in the melanization and encapsulation of certain parasites of insects (Poinar et al., 1968; and Gupta, 1979).

1.3. DEFENCE REACTIONS IN INSECTS

There are as many as three million different species of insects living in almost every habitat on earth (Williams, 1960). During their evolution and colonisation of the terrestrial niches, the insects became open to attack from predators, parasites, parasitoids and micro-organisms. Recent research indicates that they have effective defence mechanisms capable of resisting, containing and eliminating many invaders (Whitcomb et al., 1974; Ratcliffe, 1986; Ratcliffe et al., 1985, and Lackie, 1986c). These defence mechanisms include the physiochemical barriers formed by the outer tough cuticle and gut, and the internal cellular and humoral defence mechanisms (Salt, 1970). The cellular and humoral arms of insect immunity probably interact, although Rowley and Ratcliffe (1981) have stressed the need for further studies of possible functional co-operation between the two sides of the immune system.

The cellular defence reactions of insects are mainly mediated by the haemocytes and include the processes of phagocytosis, nodule formation, encapsulation, and haemolymph coagulation (Salt, 1970; Rowley and Ratcliffe, 1981). Other tissues in the insect are, however, capable of responding to invaders in a defensive manner. Kawanishi et al. (1978) described bacterial endocytosis and degradation by the lining cells of the gut. Other cells which may have a defensive role are the pericardial cells, ventral nephrocytes, diaphragm cells and garland cells (Rowley and Ratcliffe, 1981) since these may also phagocytose micro-organisms (Cameron, 1934, and Lackie, 1986).

1.3.1. Cellular Defence Reactions

1.3.1.1. Phagocytosis

Haemocytes which play an important role in the cellular defence reactions are often found in organs called phagocytic or haemopoietic organs. These organs may be diffuse and little more than accumulations of sessile haemocytes, or they may be highly organised and surrounded by a limiting membrane (Jones, 1970; Zachary et al., 1981; Nappi, 1984, and Nappi and Carton, 1986). Historically, phagocytosis was the first host defence reaction to be studied and the pioneering work of Metchnikoff (1884, 1892) led to modern-day concepts of cellular immunity. Early authors (Cuenot, 1896; Metalnikov, 1908, 1924, 1927; Holland, 1909, 1930; Metalnikov and Chorine, 1928; Cameron, 1934) mentioned that insects could withstand enormous injections of bacteria that were highly pathogenic to man. Work on phagocytosis has been carried out *in vivo* and *in vitro*. *In vivo*, most workers showed that following the injection of different particles such as carmine, polystyrene beads, Indian ink, erythrocytes, bacteria, fungi, yeast, mycetomes and protozoa, phagocytosis took place within one hour (Jones, 1962; Salt, 1970; Arnold, 1974 and Whitcomb et al., 1974). However, there is much confusion from the *in vivo* work in that the haemogram may be raised, lowered, or show no effect at all after injection with foreign particles (Jones, 1962; Wittig, 1965; Hoffman and Brehelin, 1971 and Hoffmann et al., 1974. Arnold (1974) concluded that the results of various experimental procedures on insect haemograms are often contradictory and that generalisations can seldom be made.

Phagocytosis *in vitro* has recently been studied in detail. However, there are certain disadvantages in studying phagocytosis *in vitro*, as conditions are likely to be suboptimal for maximal haemocyte activity (Ratcliffe and Rowley, 1979a). Rabinovitch and De Stefano (1970) mentioned that haemocytes respond to a greater range of test particles *in vivo* than *in vitro*. For this reason, results from *in vitro* studies should, wherever possible, be related back to the *in vivo* state.

Phagocytosis is generally considered to occur in four different stages: (a) chemotaxis, (b) attachment/recognition, (c) ingestion, (d) digestion. But many authors have mentioned that stages (a) and (d) do not always occur in insects (Rowley, 1977; Rowley and Ratcliffe, 1981; Ratcliffe and Rowley, 1983; Ratcliffe et al., 1985 and Ratcliffe, 1986.

(a) Chemotaxis

The first requisite for phagocytosis is contact between the "foreign" body and the haemocyte surface. Salt (1970) believed that such contacts are random and this view was also shared by Jones (1956), who failed to observe any movement of the haemocytes towards the foreign particles. Metalnikov (1927) mentioned that phagocytes were able to accept or reject foreign substances. On the other hand, Ratcliffe and his co-workers provided direct evidence for chemotactic responses in a range of insects used in their research Ratcliffe, 1986; Ratcloffe et al., 1976; Gagen and Ratcliffe, 1976; and Ratcliffe and Gagen, 1976a). Nappi and Stoffalano (1972a) also found that during encapsulation of

nematodes by haemocytes of Musca spp. chemotaxis may have been involved.

(b) Attachment/Recognition

Attachment of the foreign agent to the surface of the phagocyte is the end-point of recognition. The mechanisms involved in these stages have been widely studied and described in the vertebrates (Rowley, 1977; Rowley and Ratcliffe, 1980, 1981). The principal mechanism of recognition of foreignness in vertebrates is mediated by the immunoglobulins. Following the injection of foreign matter, specific antibody (eg. Opsonin) is formed which acts to identify these antigens and aid in their phagocytosis. This mechanism of recognition and attachment is called serum dependent attachment, but serum independent attachment is carried out in the absence of serum antibody (Rabinovitch, 1967, 1969). The latter mechanism is thought to be more primitive.

Invertebrates have the ability to recognise self from non-self but immunoglobulin-like molecules are absent in invertebrate body fluids. Recognition of foreignness may therefore be mediated by serum independent mechanisms or by other types of recognition factors present in invertebrate fluids (Whitcomb et al., 1974). Agglutinins which are naturally found in the body fluids of many invertebrates have been considered to act as a primitive type of "antibody molecule" (Tripp, 1966; Acton and Weinheimer, 1974).

Some workers have proposed that the role of agglutinins in the cellular defences of insects is weak as mentioned by many workers (Briggs, 1958; Feir and Waltz, 1964, and Gilliam and Jeter, 1970). On

the other hand, Scott (1971a); Lackie (1980, 1981a and 1983a); Ratcliffe (1986), and Ratcliffe and Rowley (1983) have described a positive role for agglutinins in the cellular defences of insects. In some insects antigenic challenge can cause the formation of or stimulate increased levels of agglutinins (Gilliam and Jeter, 1970, and Lackie, 1980, 1983b). Recently, many studies have been concerned with recording the immune recognition mechanisms, the presence or absence of agglutinins, the specificity of these substances; and their opsonic activity (McKay et al., 1969; Lackie, 1979, 1980, 1981, 1986b, c; Rowley and Ratcliffe, 1980, 1981; Jurinka et al., 1982; Ratcliffe and Rowley, 1983; Ratcliffe et al., 1985), but it is not yet clear whether agglutinins play a universal role in opsonisation.

(c) Ingestion

Many workers have succeeded in showing the presence of intracellular particles (see Gilliam and Shimanuki, 1967b; Rabinovitch and De Stefano, 1970, and Anderson et al., 1973a) but none witnessed the actual ingestion. Many other attempts to study this process in vitro have also failed (Akesson, 1953; Jones, 1956; Whitten, 1964, and Lea and Gilbert, 1966).

Three methods of particle uptake have been reported in insect haemocytes (Salt, 1970; Rowley and Ratcliffe, 1981, and Ratcliffe et al., 1985). These methods were as follows: Firstly by the formation of pinocytic vesicles to engulf fluid and small particles such as viruses (Leutenegger, 1967; Whitcomb et al., 1974). Secondly, by encircling particles with pseudopodia as seen in vertebrate phagocytes (Brewer, 1963; Horn et al., 1969; Jones and Hirsch, 1971, and Belcher et al.,

1973), and, finally, by close contact and spreading of the plasma membrane around the particles (Grimstone et al., 1967).

Anderson et al. (1973a); Anderson (1974); Cheng (1986) and Ratcliffe (1986) showed that many of the characteristic metabolic alterations seen in mammalian phagocytes such as increased lactase production, increased oxygen uptake and increased glucose oxidation via the hexose monophosphate pathway, were absent in the haemocytes of Blaberus craniifer. Only a general increase in the glycolysis rate occurred in these cells, during the ingestion of latex spheres

(d) Digestion

This is the final stage in the process of phagocytosis during which ingested micro-organisms are broken down by enzymes and other anti-microbial factors. Bacteria engulfed by insect phagocytes may be killed and digested, or resist attack and remain alive (Ratcliffe, 1986). Rowley and Ratcliffe (1976b) and Gagen and Ratcliffe (1976) mentioned that engulfed bacteria are destroyed and Holland (1930) found that bacilli were broken down to small granules which persisted for a long time in the cytoplasm. His studies were carried out on tubercle bacilli (Mycobacterium tuberculosis) which are not pathogenic in caterpillars. Insect phagocytes may also be used as a haven in which pathogens can develop and multiply. Schmittner and McGhee (1970) used insect haemocytes as sites of division for Trypanosoma rangeli and Crithidia spp., both of which are apparently unaffected by any antimicrobial armory present. Anderson et al. (1973a) found that some bacteria are killed by haemocytes while others remained viable.

Anderson (1974), and Anderson et al. (1973a, 1973b) in their studies on the anti-microbial system of some insects failed to demonstrate the presence of the myeloperoxidase-H_2O_2 halide system, which is found in vertebrates and some invertebrates, and they mentioned that other killing mechanisms must be present. Landureau and Grellet (1975) showed that both lysozyme and chitinase were formed in Periplaneta americana haemocyte cultures, and these enzymes may play an important role in bacterial killing.

1.3.1.2. Nodule Formation

When small doses of bacteria are innoculated into insects, they will be phagocytosed, while larger doses stimulate nodule formation (Walters and Ratcliffe, 1980). The literature describing the formation of nodules in insects presents a confusing picture. Holland (1930) and Hoffman et al (1974) believed that the clumps of phagocytosing haemocytes are encapsulated after ingestion of bacteria or other particles, and that the centres of these nodules eventually degenerate and become melanized. Salt (1970) and Rowley and Ratcliffe (1981) also described nodule formation as the clumping together of blood cells containing phagocytosed particles followed by the encapsulation of degenerated cells. But Gagen and Ratcliffe (1976) and Ratcliffe and Gagen (1976a, b) found that injected bacteria in Galleria mellonella and Pieris brassicae were rapidly removed from the circulation by entrapment in material released from some of the haemocytes, and that phagocytosis played no important role at this stage. They further showed that this material was derived from the granules in the granular cells, and that the latter stages of nodule formation were similar to encapsulation with

the formation of multi-layered cellular capsules. Ratcliffe and Rowley (1979a), and Ratcliffe (1982) believed that nodule formation occurs in two stages. In the first stage granule-containing cells randomly contact bacteria and degranulate to become sticky and form a small aggregate or clot which entraps large numbers of bacteria. Since this process is extremely rapid and can occur within 1 minute post-injection, they proposed that neither hormonal imbalances nor chemotaxis are likely to be involved. The aggregate rapidly melanizes, and the second stage takes place during which large numbers of plasmatocytes attach to form a multicellular sheath. Because this second stage is somewhat delayed and usually only involves the plasmatocytes, it has been suggested that the cells are responding to a chemotactic stimulus emanating from the granular cell/bacteria aggregate.

Walters and Ratcliffe (1980; 1983) mentioned that the extent of the host reaction depends upon the pathogenicity and mode of pathogenicity of the invading bacterium, eg. when G. mellonella larvae are injected with various doses of non-pathogenic Escherischia coli or pathogenic Bacillus Cereus, and dissected at various times post-innoculation, nodules are formed earlier and are much larger in response to the pathogen than to the non-pathogen.

1.3.1.3. Encapsulation

Encapsulation is the method by which insects deal with foreign bodies that enter the haemocoele but are too large to be phagocytosed by individual haemocytes (Rowley, 1977a; Ratcliffe and Rowley, 1979a).

The circulating blood cells of insects, the haemocytes, adhere to and form multicellular capsules around foreign objects that enter the haemocoele. The thickness of the capsule depends not only on the species of insect but also on the nature of the object encapsulated (Lackie, 1983b and 1986c). Lackie et al. (1985) believed that for encapsulation to occur, there must be several important changes in the behaviour of haemocytes, involving not only cell substratum adhesion, but also subsequent recruitment and adhesion of cells to each other. Finally, recruitment must stop and the capsule must be consolidated.

Capsules are formed in insects against a wide range of non-biological and biological objects, including Araldite (Grimstone et al., 1967), cellophane fragments, cat gut, cotton (Brehelin et al., 1975), latex (Lackie, 1976), nylon fibres (Salt, 1965), biological implants (Schmit and Ratcliffe, 1977), acanthocephalan larvae (Robinson and Strickland, 1969) and insect parasitoids (Salt, 1970). However autologous implants do not result in an encapsulation response (Scott, 1971a) thereby demonstrating the capacity of the haemocytes involved for "self" - "non-self" discrimination.

Basically there are two types of capsules, cellular and humoral, but encapsulation results from an interaction of the cellular and humoral components of the haemolymph (Ratcliffe, 1982). What determines the type of capsule formed is probably the number of blood cells available for the host response (Salt, 1963; Poinar and Leutenegger, 1971; Gotz et al., 1977; Poinar et al., 1979).

Studies on the structure and mode of formation of cellular capsules indicate that the process may be similar to nodule formation and involve the interplay of different haemocyte types (Sato et al., 1976 and Schmit and Ratcliffe, 1977, 1978). Schmit and Ratcliffe (1977) showed that cellular encapsulation is a two-stage process. During the first phase, granular cells rapidly lysed on the surface of the implant and inducing a localised clot-like reaction. The second stage starts after 20 minutes and this involved the specific attachment of plasmatocytes to regions of the implant on which granular lysis had occurred. Some melanization of the inner layers was frequently observed during cellular encapsulation (Ratcliffe, 1986; Ratcliffe and Rowley, 1979a and Ratcliffe et al., 1985).

Humoral encapsulation consists of the laying down of melanin on the surface of the implant (mainly parasites) so that it becomes surrounded by a pigmented sheath (Gotz, 1969). Bronskil (1962) mentioned that humoral encapsulation has been observed to occur in two stages, first a thin melanized sheath is formed, and the second step is the deposition of melanin particles. The cause of death of parasites inside capsules is not exactly known, but it may result from asphyxiation, inanition or from some active component of the phenoloxidase system. (Singh, 1978) and Beresky and Hall (1977) proposed that melanin toxicity is the major cause of death.

1.3.1.4. Haemolymph Coagulation

One of the major defensive barriers of multicellular organisms against pathogenic attack is provided by the skin and/or cuticle, When

this first line of defence is breached, it is important to restrict the access of pathogens to the interior of the body. One way in which this may be achieved is to seal the wound with a coagulum. This process has been fully reviewed elsewhere (Gregoire, 1953, 1954, 1970, 1974; Crossley, 1975 and Rowley and Ratcliffe, 1981). Gregoire referred to four methods of haemolymph coagulation which he termed Patterns I - IV. Pattern I consisted of coagulation brought about exclusively by the release of products from cystocytes/coagulocytes; Pattern II involved the formation of strand-like material by certain haemocytes to form a network; Pattern III was a mixture of both I and II, while Pattern IV consisted of coagulation not involving the haemocytes. Ratcliffe (1982) believed that clotting occurs and is mediated by materials discharged from granular cells and/or cystocytes which probably interact with plasma proteins to induce gelation. Little is known about the initiation of haemolymph coagulation or of the changes occurring in the cell types involved. Biochemical events occurring during this process and the actual stimulus for granule discharge from the haemocytes are also poorly understood (Rowley, 1977b). It has been argued (Ratcliffe and Rowley, 1979, and Bohn, 1986) that the clotting substances involved in coagulation reactions are also important immunologically in phagocytosis, nodule formation and encapsulation.

1.3.2. The Humoral Defence Mechanisms

Both natural (innate) and induced humoral antibacterial activity have been demonstrated in many insect species (Briggs, 1958; Stephens, 1959; Wagner, 1961; Gingrick, 1964; Heimpel and Harshbarger, 1965;

Chadwick, 1967, 1975; Hoffman, 1980; Lackie, 1980, and Walters and Ratcliffe, 1983).

(a) Naturally occurring humoral mechanisms:

The first antibacterial factor to be identified in insect haemolymph was lysozyme and it has been claimed that this enzyme is the main antibacterial factor responsible for immunity of vaccinated insects (Mohrig and Messner, 1968). Boman (1982, 1986), however, pointed out that insects can eliminate many lysozyme-resistant bacteria. Stephens (1959) mentioned that only three types of soluble factors which are functionally comparable to mammalian antibodies have been reported in insects, namely: agglutinins, lysins and antitoxins. Many authors agree with this view, among them Glaser (1918); Paillot (1920); Chorine (1929); Briggs (1958), and Chadwick (1967 & 1975). Ingram et al. (1983, 1984) and Molyneaux et al. (1986) reported that the haemolymph of insects contains molecules which agglutinate mammalian erythrocytes (haemagglutinins); opsonins which stimulate phagocytosis and bactericidins (including lysozyme). Gotz (1986) proposed that humoral reactions also include humoral encapsulation (which consists of the deposition of a quick hardening material on foreign surfaces without the visible participation of blood cells) and the formation of antibacterial proteins and other antibiotic factors. Precipitins or complement-like factors are rare or absent in invertebrates (Anderson et al., 1972, and Aston et al., 1976). But widely distributed antibacterial factors are lysozyme, melanin and agglutinins (Rowley, 1977a).

1.3.2.1. Lysozyme

Lysozyme is an enzyme widely distributed in bacteria, plant and animal life which acts by splitting the 1-4 linkages between N-acetyl muramic acid (NAMA) and N-acetyl glucosamine (NAG) (Rowley, 1977a).

Boman (1982, 1986) suggested that lysozymes could be considered as ideal immune substances for an insect because they have the widest action against foreign intruders without causing any self-destruction. In the moth Cecropia, there are two types of lysozyme called cecropins and attacins. Boman et al. (1985, 1986) has identified three different cecropins (A, B and D) and at least two different attacins in Cecropia but differences in function between these multiple antibacterial factors have not been described. Mohrig et al. (1970) recorded lysozyme in the haemolymph of the wax-moth, G. mellonella, and Mohrig and Messner (1968) have also found it in 24 other insect species. However, Landureau and Jolles (1970); Landureau et al. (1972); and Bernier et al. (1974) have shown that in the cockroach P. americana, the lysis of Micrococcus lysodeikticus is caused not only by lysozyme, but also by a closely related enzyme, chitinase. It has been suggested that the enzyme activity found in the haemolymph of insects may be partially derived from the haemocyte (Cheng et al, 1975; Landureau and Grellet, 1975, and Ratcliffe, 1986).

1.3.2.2. Melanin

Melanin production occurs both in nodule formation and encapsulation and plays an important role in combating invading parasites and micro-organisms (Taylor, 1969b; Brennan and Cheng, 1975). Melanin is a black pigment supposedly derived solely from precursors

dissolved in the haemolymph. Biochemically, it is a derivative of various aromatic quinones formed by the polyphenoloxidase system which occurs in both the haemocytes and haemolymph (Rowley, 1977a).

In some insects, melanotic crusts are deposited on the surface of foreign objects without visible participation of blood cells. This type of defence reaction has been called humoral or melanotic encapsulation (Gotz, 1986a; Gotz and Boman, 1985). Melanization of the nodules or capsules becomes obvious only hours after the onset of encapsulation. The first area of melanization is always the surface of the foreign object, or the centre of the nodule with its aggregation of granulocytes and foreign particles. The melanization process can continue for days, expanding continuously from the centre to the middle and outer layers of the capsule (Gotz, 1986a).

The prophenoloxidase activity system is a complex cascade of enzymes and other factors that are ultimately responsible for the initiation of melanin synthesis by the host (Soderhall and Smith, 1986). However, the exact relationship between melanin synthesis and non-self recognition remained a matter of speculation until Pye (1974) and Soderhall (1982) demonstrated that yeasts initiated melanization reactions in arthropod haemolymph by activating prophenoloxidase, the key enzyme in the melanization reactions.

1.3.2.3. Agglutinins

Agglutinins (or lectins) have been suggested to play a role in immunological defence systems as molecules which may cause clumping of viruses, bacteria and cellular micro-invaders (Yeaton, 1981; Ingram et

al., 1983, 1984; Molyneaux et al., 1986; and Renwrantz, 1986). It is also supposed that lectins precipitate soluble glycoconjugates and bind to determinant carbohydrate moieties of membrane glycoproteins or glycolipids thus causing agglutination of the respective cells. If lectin induced binding of bacteria to the surface of haemocytes is followed by their ingestion, all requirements for calling the lectin an opsonin are fulfilled. Opsonizing molecules are defined as humoral factors which facilitate binding of foreign particles to, and promote their ingestion by, phagocytes (Ibrahim et al., 1984; and Ratcliffe et al., 1985; Renwrantz, 1986; Renwrantz et al., 1981;

Only recently has definitive proof for the role of agglutinins as recognition molecules in invertebrates been provided (Ratcliffe, 1986). Purified molecules of agglutinin enhanced the phagocytic uptake of yeast cells by the haemocytes of the bivalve mollusc, Mytilus edulis (Renwrantz and Stahmer, 1983). In insects many authors have reported a role for agglutinins in the recognition processes (Yeaton, 1980; Lackie, 1981b, 1983a; Komano et al., 1983; Komano and Natori, 1985, and Ratcliffe et al., 1985), and the possible role of agglutinins in opsonization has been referred to in an earlier section.

(b) Induced humoral mechanisms:

Humoral immunity can be induced in insects by an injection of either live, non-pathogenic bacteria or heat-killed pathogens (Gotz and Boman, 1985). Immunisation of insects usually gives short-term protection against further infection. The process of the haemocytic response enhancement has been likened to kicking a hive of bees twice,

in that the first kick would so infuriate the colony that its response to the second would be massive and instantaneous (Lackie, 1986b). A number of authors have shown that immunization of arthropods with bacteria, yeasts or their cell wall products, confers temporary immunity on the host (Stephens, 1962; Evans et al., 1968; McKay and Jenkin, 1970; Hultmark et al., 1980). However, the question of how insect immunity relates to lectin-mediated recognition remains something of an enigma. Komano et al. (1980) have succeeded in inducing lectins specific for D-galactose in Sarcophaga peregrina larvae by injury to the body wall. In many cases, immunization has been found to lead to elevated bactericidal or lysozyme activity in the blood, but the way in which this is achieved in most insect species is unknown (Soderhall and Smith, 1986). Immunization of G. mellonella larvae with various antigens has been shown to increase the lysozyme levels in the haemolymph (Mohrig et al., 1970). Mohrig and Messner (1968) believed that in G. Mellonella the rise in the lysozyme concentration was completely responsible for the increased immunity, while Chadwick (1970) proposed that other factors were responsible for increased immunity. Hoffman (1972, 1980), and Hoffman et al. (1974) have reported that the Orthopteran Locusta migratoria can be efficiently protected against lethal doses of Bacillus thuringiensis by injections of low doses of the same pathogen. However, Walters and Ratcliffe (1983) have observed that bacteriostatic and bactericidal factors can be induced in insect larvae by immunization with pathogens, non-pathogens or even saline.

Despite the wealth of information that is accumulating concerning different aspects of insect haemocytes and immunity, there have been few systematic studies concerning the ontogenetic emergence of immunological

function in different insect species. Such studies may be of value not only in understanding developmental aspects of invertebrate immunity, but may also serve as a basis for the development of biological control programmes. In gregarious or swarming insects such as locusts, for instance, it may be possible to expose them to pathogens at immunologically vulnerable stages of their life cycle.

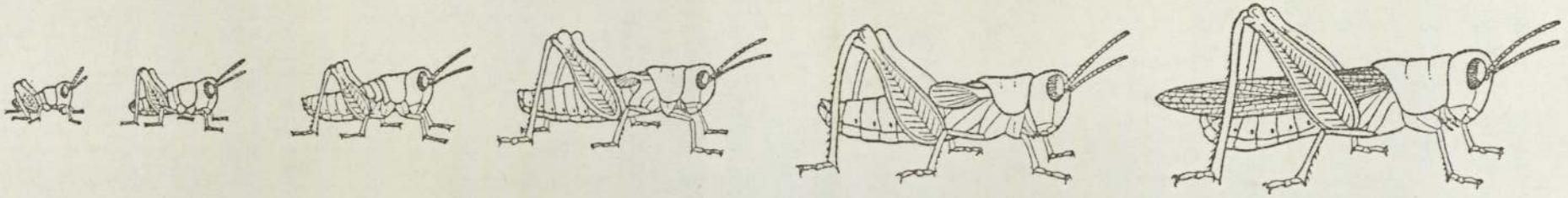
The work presented in this thesis has been directed towards studying the emergence of immunological function in two species of locust, Locusta migratoria (the migratory locust) and Schistocerca gregaria (the desert locust). Both species are of considerable economic importance and, in addition, are well-suited to immunological studies because of their relatively large size and ease of maintenance in the laboratory. A number of assay techniques have been employed in order to study a range of immunological reactions in immature and adult insects of both species (see Table 1). Chapter Two identifies and describes the blood cell types in both species of locust at each developmental stage, and also monitors changes in the relative proportions of blood cell types following antigenic challenge. Chapter Three investigates the ontogenetic emergence of agglutinins following challenge with foreign erythrocytes and bacteria. In Chapter Four the phagocytosis of a range of test particles by haemocytes and haemopoietic tissue of different developmental stages is examined. Chapter Five investigates the possible chemotactic and chemokinetic effects of antigens and soluble products of cells involved in the encapsulation response.

Stage	Duration	Mean Body Length/cm		Mean Body Weight/gm	
		L. migratoria	S. gregaria	L. migratoria	S. gregaria
Eggs	2 weeks	0.8 - 1	0.8 - 1	-	-
1st instar nymph	5-days *	0.5	0.52	0.017	0.021
2nd instar nymph	4-days *	1.2	1.3	0.046	0.048
3rd instar nymph	4-days *	2.1	2.4	0.098	0.123
4th instar nymph	5-days *	2.7	2.9	0.220	0.331
5th instar nymph	8-days *	3.4	3.8	0.684	0.703
Adult male	-	6	6.2	0.908	1.360
Adult female	-	6.1	6.32	1.343	1.832

Table 1 Life cycle of Locusta migratoria and Schistocerca gregaria showing size and duration of different nymphal instars.

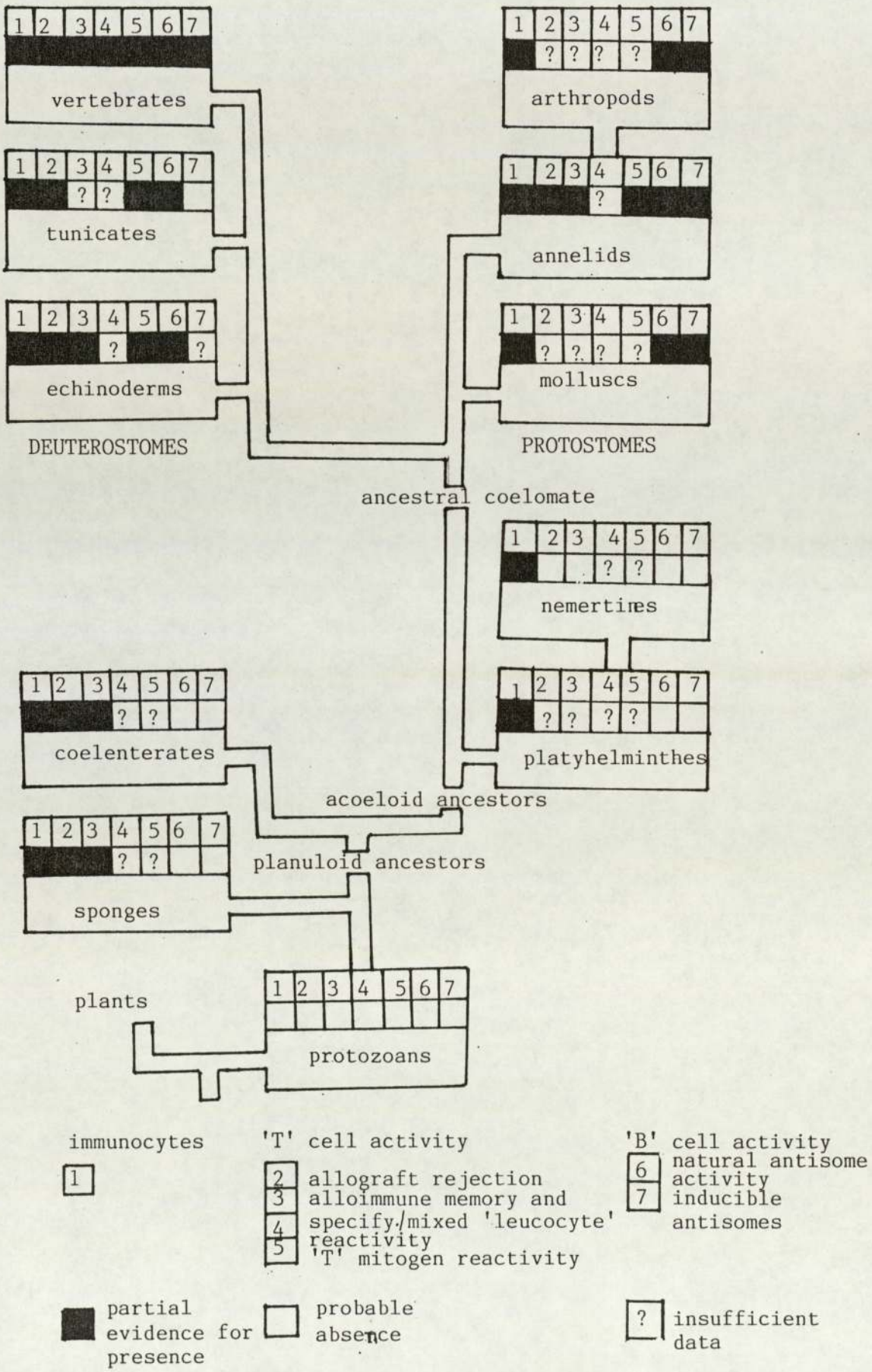
Data taken from Webley, 1951, Mathur and Soni, 1937 and Buchsbaum, 1976.
* moulting at the end of the instar.

Generalised diagramatic representation of different developmental stages of locusts



1st instar 2nd instar 3rd instar 4th instar 5th instar Adult

Diagrams from Buchsbaum, 1976


1 2 3 4 5 6 7
vertebrates
1 2 3 4 5 6 7
? ? ? ?
arthropods
1 2 3 4 5 6 7
? ?
tunicates
1 2 3 4 5 6 7
?
annelids
1 2 3 4 5 6 7
? ?
echinoderms
1 2 3 4 5 6 7
? ? ? ?
molluscs
DEUTEROSTOMES
PROTOSTOMES
ancestral coelomate
1 2 3 4 5 6 7
? ?
nemertines
1 2 3 4 5 6 7
? ? ? ?
platyhelminthes
1 2 3 4 5 6 7
? ?
coelenterates
acoeloid ancestors
1 2 3 4 5 6 7
? ?
sponges
planuloid ancestors
plants
1 2 3 4 5 6 7
protozoans
immunocytes
1
'T' cell activity
2 allograft rejection
3 alloimmune memory and
4 specify./mixed 'leucocyte'
5 reactivity
'T' mitogen reactivity
'B' cell activity
6 natural antisome
7 activity inducible
antisomes
partial evidence for presence
probable absence
? insufficient data

CHAPTER II

AN ONTOGENETIC STUDY OF CHANGES IN CIRCULATING HAEMOCYTE LEVELS FOLLOWING ANTIGEN CHALLENGE

2.0 Introduction

Insect haemocytes may be free, sessile, or arranged into aggregates of differing complexity termed haemopoietic or phagocytic organs (Jones 1970). Extensive information is available on the form and function of insect blood cells (Gupta 1979; Rowley and Ratcliffe 1981; Ratcliffe 1986; Ratcliffe et. al. 1985 and Molyneux et. al. 1986). However, there have been, and still are, many problems associated with the classification of insect blood cells (Ratcliffe, 1986). This situation was partially alleviated by Jones (1962) whose scheme categorized nine haemocyte types and by Price and Ratcliffe (1974) who identified six basic types of cells. Despite these advances, many difficulties still remain and additional work in this field is urgently required (Brehelin and Zachary, 1986).

Recent studies on ultrastructure, function, histology, separation and modifications of insect haemocytes have been carried out by many authors, among them: Jones (1962); Hoffmann (1972); Hall (1983); Brehelin and Zachary (1983); Essawy et. al. (1985); Mead and Ratcliffe (1985); Ratcliffe (1986); Ratcliffe and Rowley (1979a,b); Ratcliffe et. al. (1985); Lackie (1986c); Lackie et. al. (1985) and Brehelin and Zachary (1983, 1986). In addition, there have been numerous studies of total haemocyte numbers in many orders of insects, including those by Yeager (1945); Smith (1938); Arvy et. al. (1948); Jones (1950, 1956, 1962, 1967b and c); Patton and Flint (1959); Jones and Liu (1961); Collin (1963); Crossley (1964); Arnold

(1966); Schwartz and Townshend (1968); Salt (1970); Bahadur and Pathak (1971); Vinson (1971); Nappi and Stoffolano (1972b) and Arnold and Hinks (1976). For the two species studied here, the total haemocyte counts of nymphal instars and adult locusts have been examined by Mathur and Soni (1937) for Schistocerca gregaria and by Webley (1951) for Locusta migratoria. Hoffmann (1972) also investigated the normal total haemocyte numbers of adult and 5th instars and examined the changes in these numbers after irradiation of the haemopoietic tissue of Locusta migratoria.

The numbers of circulating cells in insects vary from time to time. The highest values are obtained after injury and haemorrage; in insects attacked by parasites; and particularly during ecdysis. Numbers are also affected by sampling technique, age, stage and the physiological status of the insect (Salt, 1970). Whitcomb et. al. (1974) mentioned that total haemocyte counts range from less than 10,000/mm^3 to 100,000/mm^3 and in a few cases they may reach up to 16 millions/mm^3 depending on the species studied.

Variations in haemocyte number relating to sex and developmental stage have been described by Webley (1951) and Mathur and Soni (1937) in Schistocerca gregaria and Locusta migratoria respectively. They also mentioned that there is a progressive increase in haemocytes during larval life and after each moult, the total numbers are reduced and then increase again as growth is renewed.

Differential haemocyte counts (DHC) in different insects have also received considerable attention (Jones, 1950, 1956, 1967 b,c; Jones and Liu, 1961; Collin, 1963; Lea, 1964; Arnold, 1966; Nappi,

1970; Nappi and Stoffolano, 1972a, b; Essawy et. al. 1985a; Lackie et. al. 1985).

Metalnikov (1927) mentioned that changes in circulating cells depend on the quality and quantity of injected substances. Jones (1950) found that the DHC changed after injection of distilled water into Tenebrio (mealworm) larvae and the mitotic count also increased. On the other hand, Wittig (1966) reported that there was not change in DHC following injection of mealworm larvae with nonsterile distilled water. Much work has been done on changes in the haemocyte picture after injection of ink or carmine often with conflicting results. Rooseboom (1937) found no changes in the haemocyte profile after injection of chinese ink in Carausius, but Toumanoff (1930) in Apis and Cameron (1934) in Galleria, noticed significant alterations following challenge. After injection of sheep erythrocytes and latex particles, Galleria larvae died within 24-48 hr, but changes in DHC were observed after injection of larvae with indian ink (Werner and Jones, 1969).

In view of the variations recorded above, it is obviously important before embarking on any detailed study of insect cellular defense reactions, to clarify the cell types present in the insects under study. In this chapter the blood cell types in different stages are established, and changes in the haemocyte populations following exposure to two different antigens (sheep red blood cells (SRBC) and Escherichia coli) are studied. These studies were carried out on the third, fourth and fifth nymphal instars and adults of both locust species.

In addition to cirulating haemocytes there are, in insects, permanent aggregations of cells which form distinct organs of more or less constant position and form. These collections of cells are often regarded as blood-forming or haemopoietic organs (Wigglesworth, 1959 and Rowley and Ratcliffe, 1981). Hoffmann (1970) graded insect haemopoietic organs into three types which he believed represented possible evolutionary stages in their development. The first type is composed of dissociated masses of haemocytes including cells undergoing mitosis. The second category includes groups of cells forming a diffuse tissue with little signs of differentiation into distinct regions and unbounded by either a limiting membrane or a connective tissue sheath. Examples of this type of haemopoietic organ include those of L. migratoria (Hoffmann, 1970 and Hoffmann et. al. 1974). which have been shown to contain reticular cells, be similar to those of vertebrate lymphatic tissue (Ratcliffe, 1986). The cells are loosely aggregated into vast networks which divide and form haemopoietic foci which in turn mature to form the haemocytes (Rowley and Ratcliffe, 1981). The third type of haemopoietic organ is more highly developed, showing some signs of differentiation into various zones and is bounded by a limiting membrane.

The importance of the haemopoietic organs in haemocyte production has been demonstrated by irradiating these organs (Hoffmann, 1972; Zachary and Hoffmann, 1973), ligaturing the haemopoietic tissue (Hinks and Arnold, 1977), or by surgical removal (Beaulaton, 1968). These modifications generally cause a great drop in the number of circulating haemocytes and testify to the haemopoietic role of these

organs. The haemopoietic tissue of L. migratoria and S. gregaria consists of several cell layers situated on the upper surface of the dorsal diaphragm, mainly in the first four abdominal segments. The cell layers are not surrounded by a limiting membrane (Hoffmann, 1970, 1972; Zachary et. al. 1981).

In locusts, the aggregations of cells along the heart continue to proliferate throughout the life of the insect producing the different types of haemocytes (Gupta, 1979). Jones and Liu (1961) showed that mitosis alone could account for the maintenance of haemocyte numbers, at least under normal conditions. Presumably after the formation of haemocytes from the mesodermal elements these cells divide and differentiate during the remainder of the insect's life. Hoffmann (1973) stressed the importance of the haemopoietic organs alone in maintaining the normal haemogramme and rarely mentioned the role of mitosis in peripheral cells. However, Rowley and Ratcliffe (1981) proposed that both processes probably operate, depending on the species examined and the stage in the life cycle.

In view of these uncertainties regarding the proliferative potential of cells in the circulation and haemopoietic tissues, some preliminary autoradiographical studies were also employed here in order to provide information concerning proliferative events in these two cell pools following antigenic challenge.

2.1 Materials and Methods

2.1.1 Insects

The insects used in this study were the 3rd, 4th, and 5th nymphal instars and the adult stages of Locusta

migratoria and Schistocerca gregaria. Both species were reared in standard locust cages (Phillip Harris Ltd) large enough for up to 300 adult locusts. The insects were maintained in a thermostatically controlled hot room at a temperature of 28-33° C, with a day and night (light and dark) cycle of 8 and 16 hours respectively. Both species of locust were fed on fresh grass and spring cabbage three times a day and, over the week end, dry wheat bran was provided as additional food. Water-soaked cotton wool was placed in each cage to maintain the correct level of humidity.

2.1.2 Antigens preparation

The antigens used in this study were sheep red blood cells (SRBC) (Gibco Ltd) and E. coli bacteria (obtained form the microbiology laboratory, Aston University). SRBC were prepared by washing Alsever's-stored sheep erythrocytes three times in 0.9% saline (by centrifugation at 1500 rpm for 10 minutes) and resuspended in fresh saline as a 1% v/v suspension.

E. coli bacteria were removed from nutrient agar by repeated washing with sterile saline and suspended in 0.9% saline solution. Bacterial counts were made with a Thoma counting chamber and the concentration of bacteria used in this study was 9×10^5 organisms/ml.

2.1.3 Antigen injection

Following surface sterilisation with absolute alcohol, all injections were performed between the third and fourth sternites of insects. All insects were injected with a Microtitre Syringe (S.G.E. Type A.R.N.) fitted with a guage needle. All antigen

injections were in 10 µl volumes for adults and 5th instar nymphs, and in 2 µl for the 3rd and 4th nymphal instars. Control insects were injected with equivalent volumes of 0.5% saline solution.

2.1.4 Collection of haemolymph

Locusts were precooled in the fridge at 4°C for 20 minutes to slow down coagulation of the haemolymph and to reduce their activity. They were bled by leg amputation at the coxa according to Hoffmann et. al. (1974). Drops of haemolymph were collected on individual slides.

2.1.5 Differential blood cell counts

Haemolymph was allowed to fall onto a clean microscope slide and a smear was made by drawing a second slide across the first one at a 45° angle. The smear was allowed to air-dry and was stained using Leishman's stain as described by Pappenheim (1914). Stained smears were examined by light microscopy under oil immersion, and a differential count was obtained by examining approximately 200 cells per slide (Salt, 1970).

Fresh slide preparations were also examined and found to be superior to stained preparations. Briefly, one drop of haemolymph was dropped over one drop of paraffin oil on a glass slide and then a drop of 0.5% saline solution was added and the cells left to settle for 3-5 minutes. These preparations were observed and photographed under phase contrast using a Zeiss photomicroscope.

Differential blood cell counts were carried out by examining fresh haemolymph samples collected from five adult or fifth instar locusts. For third and fourth instar nymphs, blood was pooled from

10-15 individuals and five samples of pooled blood were examined. Differential counts were obtained by examining 250 cells per sample and calculating the individual percentages of coagulocytes, granulocytes and plasmatocytes. Following antigenic challenge, differential haemocyte counts were performed on each developmental stage at intervals upto seven days post injection.

2.1.6 Autoradiographic studies

Only adult L. migratoria and S. gregaria were used in this part of the study. Each insect was injected with 10 μl of E. coli suspension as described earlier. Following antigenic challenge, groups of immunised locusts received 5 μl of tritiated thymidine (S. A. 5 ci/mmol) after the method of Nath and Mehrotra (1982). Radioisotope was administered at one, two, four or seven days post antigen challenge, and insects were sacrificed 24 hours after tritiated thymidine injection. Haemolymph smears were air-dried prior to fixation and staining. Haemopoietic tissues were fixed in Bouin's fixative and, after wax embedding and sectioning, autoradiographs of both blood smears and haemopoietic tissue sections were prepared by the liquid emulsion technique of Bancroft and Stevens (1982).

2.2. Results

2.2.1 Differential haemocyte counts

Differential haemocyte counts (expressed as percentage of total haemocytes) for control animals of each developmental stage are illustrated in Figures 2.1 and 2.2. For both species, coagulocyte numbers were found to be highest followed by granulocytes and

plasmatocytes in descending order. These ratios were similar in both immature and adult stages.

Following antigenic challenge with SRBC and E. coli, there were a number of marked changes in the differential haemocyte counts of immature and adult stages in both species. These changes are illustrated in Figures 2.3 to 2.5 and the data from which these figures are derived are presented in Tables 2.1 to 2.4 (Appendix). There was a marked similarity in the pattern of cellular changes in both locust species following challenge with both antigens. For ease of interpretation, changes in granulocyte numbers for each developmental stage and each antigen have been presented together in Figures 2.3.a to 2.3.d. Following antigenic challenge, there was a general increase in the percentage of granulocutes in the haemolymph regardless of the antigen employed or the developmental stage studied.

The increase in granulocyte numbers following antigenic challenge was matched by a corresponding decline in coagulocyte numbers. This decrease is illustrated in Figure 2.4.a to 2.4.d where it is apparent that the fall in coagulocyte numbers is seen in all developmental stages following challenge with both SRBC and E. coli. Both the changes described above were often more marked (especially during the early stages of the response) in third and fourth instar nymphs than in older stages.

Changes in plasmatocyte numbers following antigenic challenge (Figs. 2.5.a to 2.5.d) were less consistent than those observed for coagulocytes and granulocytes. Plasmatocyte numbers in fifth instar

nymphs and adult locusts of both species remained relatively stable over the period of study sometimes increasing and, in other cases, declining. In third and fourth instar nymphs, however, there was in all cases except one, (following injection of fourth instar nymphs of both locust species with E. coli (Figs. 2.5.c and 2.5.d) an increase in plasmatocyte numbers following antigen injection. This increase was sometimes prolonged (e.g. following injection of third instar S. gregaria with SRBC (Fig. 2.5.b) but in other cases was transient and plasmatocyte levels rapidly declined (e.g. following injection of third instar L. migratoria with SRBC (Fig. 2.5.a).

Examples of granuloctes, coaguloctes and plasmatocytes as seen under phase contrast microscopy are shown in Figs. 2.6, 2.7 and 2.8. Examples of autoradiographs of haemopoietic tissue following antigenic challenge are presented in Figure 2.9 which illustrates the presence of heavy labelling in this region. The amount of label present made it difficult to discern whether the silver granules were associated with individual cells. But there was a much lower level of label in the haemopoietic tissue of locusts which had not previously received antigen injections. Examination of haemocyte smears prepared for autoradiography failed to reveal evidence of labelling in circulating blood cells of control or experimental animals.

2.3 Discussion

The haemolymph of insects contains large numbers of haemocytes which either float freely in the circulation or are attached to various organs and tissues (Rowley and Ratcliffe, 1981; Ratcliffe et. al., 1985; Brehelin and Zachary, 1986). These haemocytes are

of haemocyte types present in the haemolymph was similar in controls of all developmental stages examined.

The second part of the work presented in this chapter examined changes in differential haemocyte counts following antigenic challenge. For the blood picture of an insect to be as complete as possible, total haemocyte counts and blood volume studies are also helpful, (Shapiro, 1979) but these were beyond the scope of the present work. It must be remembered therefore, that the changes reported here are relative changes in cell number and may not necessarily reflect total numerical changes in different haemocyte populations. Thus, a decline in one population (e.g. coagulocytes as seen here) may produce an apparent increase in another population (granulocytes) even though the total number of the latter remains constant. However, it was felt that changes in differential haemocyte counts may give some clues to the function of different insect haemocyte populations.

Following challenge by SRBC or E. coli there was a marked change in the relative percentages of circulating haemocytes in all developmental stages. From being the most common blood cell type in control locusts, the percentage of coagulocytes declined markedly following immunisation and the granulocytes took over as the dominant cell species. These findings are consistent with earlier reports of increases in granulocyte numbers following injection of various antigens and foreign particles into a range of insect species. Thus, granulocyte numbers increased in Tenebrio following injection of Indian ink (Jones and Tauber, 1952); in Galleria following injection of latex (Wittig, 1966); in Pieris after

injection of latex particles and Indian ink (Tokada and Kitano, 1971) and in Apis after injection of starch or red blood cells (Werner and Jones, 1969).

In the studies on Galleria and Apis the authors (Wittig, 1966 and Werner and Jones, 1969) attributed the increase in granulocyte numbers to their involvement in phagocytosis of foreign particles. However, Takado and Kitano (1971) working with Pieris reported that, although granulocyte numbers increased following challenge with latex or Indian ink, the primary phagocytic cells were the plasmatocytes. There may therefore be differences in function between the same cell type in different insect species (See also Brehelin and Zachary, 1986). Work in later chapters of this thesis has examined the functional properties of locust haemocytes in more detail.

Metalnikov (1927) concluded from his extensive studies that each excitant provoked a specific reaction among the haematocytes. However, Jones (1950) later argued that changes in the haemogram may result from a general physiological response to any excitatory agent. This latter view is certainly supported by the studies reported here where injection of SRBC or E. coli in to both species of locust produced a similar pattern of changes in the differential haemocyte count of all developmental stages. Changes in circulating haemocyte numbers may result from a variety of factors which interact in a dynamic fashion. Hoffman (1972a) and Hoffman and Porte (1973), reported that, following antigenic contact, immunological reactions may lead to the death of haemocytes which are involved in lysis and melanisation of foreign material. In

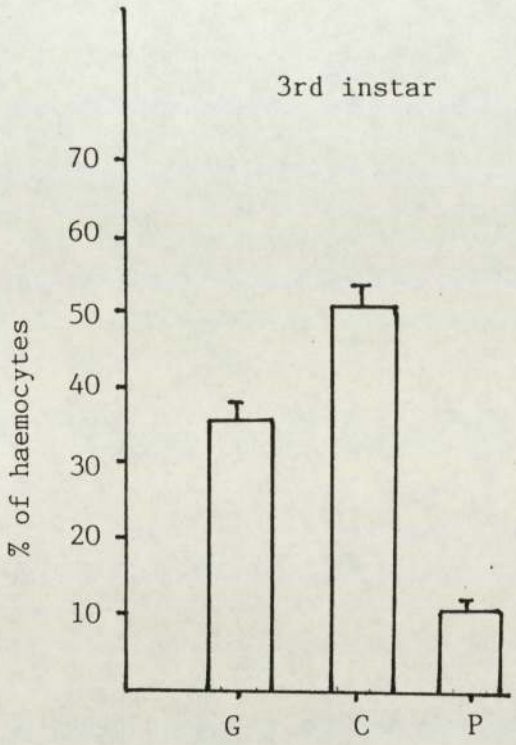
addition, they also suggested that haemopoietic tissues (which under normal conditions are responsible for the production of blood cells) may become involved in antigen uptake and temporarily suspend haemopoietic activity. Thus the death of immunologically reactive cells and the temporary block of haemopoietic functioning, may help to explain the decrease in coagulocyte numbers reported here. The decline in coagulocyte numbers could also be due to changes in their circulatory behaviour one possibility being that, following contact with antigen, they cease to circulate in the blood and become trapped in the tissues. Similar findings have also been reported by Abu-Hakima and Faye (1981) for Hyalophora cercopia haemocytes.

The changes in plasmatocyte numbers reported here were variable and unpredictable, there being non consistent pattern of increase or decrease in their relative numbers following antigenic challenge. These findings are difficult to explain in the light of their known function, since plasmatocytes are thought to be involved in phagocytosis of foreign particles and some related changes in numbers might have been expected (see Chapter 4 also). However, it is apparent from the studies of earlier workers that there is not always a direct or obvious correlation between changes in circulating cell numbers and the known function of insect haemocytes (Takado and Kitano, 1971).

In this and other studies, there are clearly changes in the relative numbers of circulating haemocytes following antigenic challenge. However, there is still considerable uncertainty about the functional significance of these changes. Rowley and Ratcliffe

(1981) mentioned that no clear picture has yet emerged and, furthermore, the factors controlling the production and differentiation of the various haemocyte types are unknown. In order to determine whether or not antigenic challenge provokes mitosis in circulating haemocyte populations or in haemopoietic tissues, some preliminary autoradiographical studies were carried out here. The results indicate that circulating haemocytes did not take up tritiated thymidine following antigenic challenge, but that some isotopic activity was located in the haemopoietic tissue. This may be indicative of proliferative activity in direct response to antigenic challenge, or may result from increased haemopoietic activity which is needed to replace peripheral cells which are lost during the course of an immune response. Further studies are required to clarify this issue.

In summary, the work presented in this chapter has revealed that the basic pattern of haemocyte cell types is established early in life in both L. migratoria and S. gregaria. Furthermore a similar pattern of changes in differential haemocyte count was observed in all developmental stages following challenge with two different antigens. Work presented in later chapters will address the issue of whether these similarities in differential haemocyte counts are reflected in terms of immunological function throughout ontogeny.


3rd instar
% of haemocytes
70
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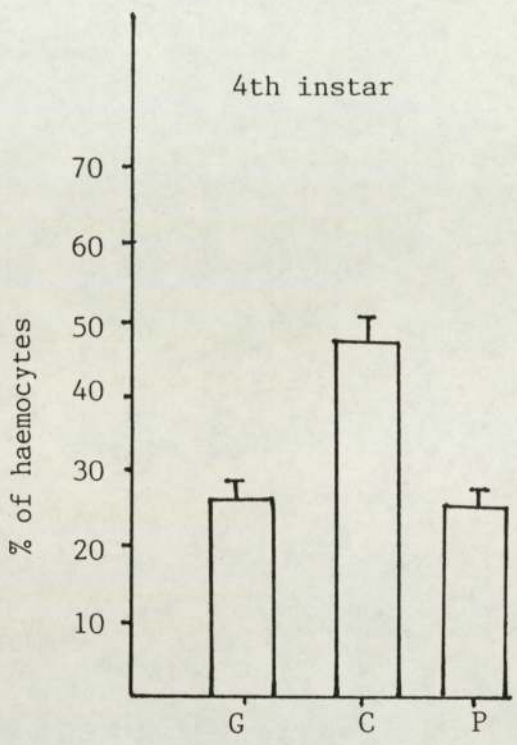
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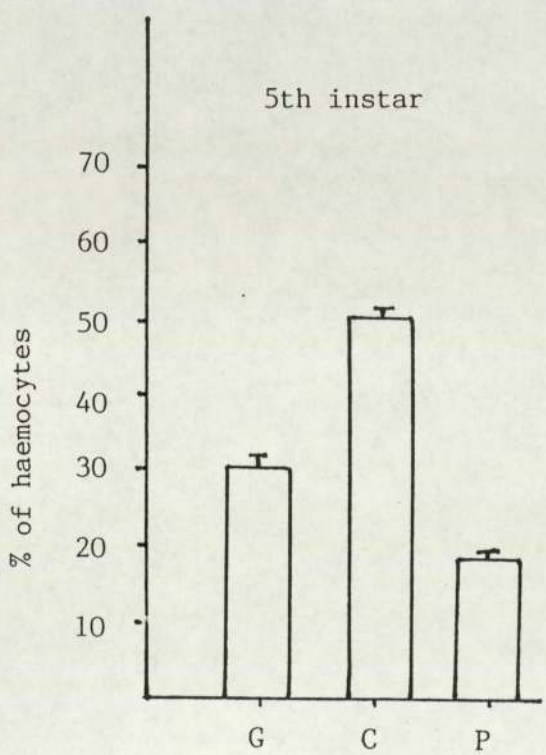
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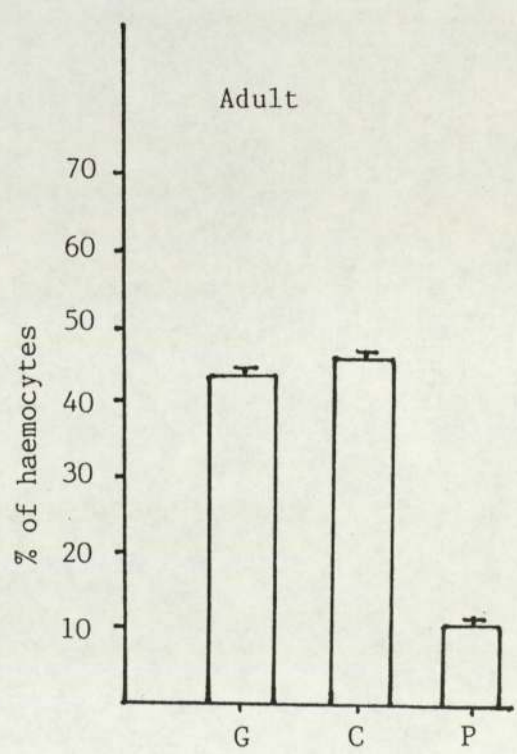
Adult
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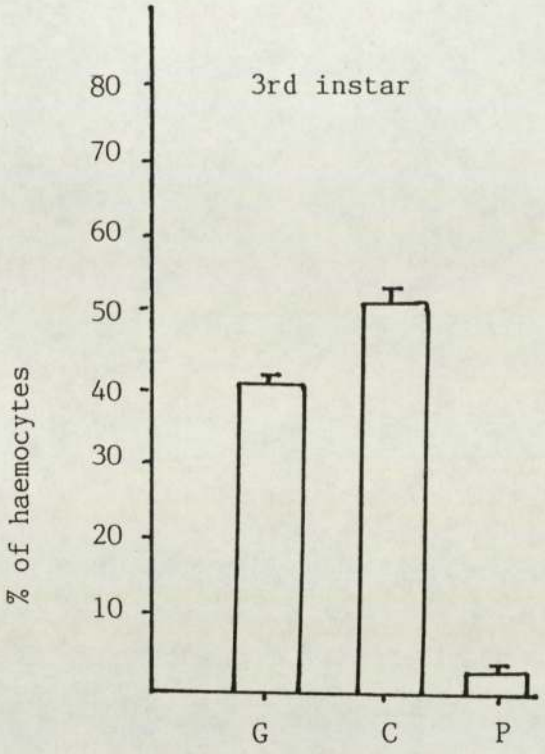
3rd instar
% of haemocytes
80
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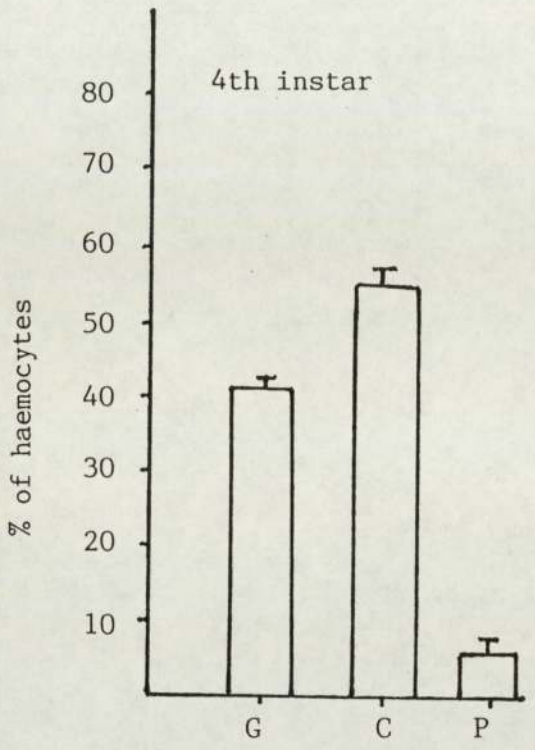
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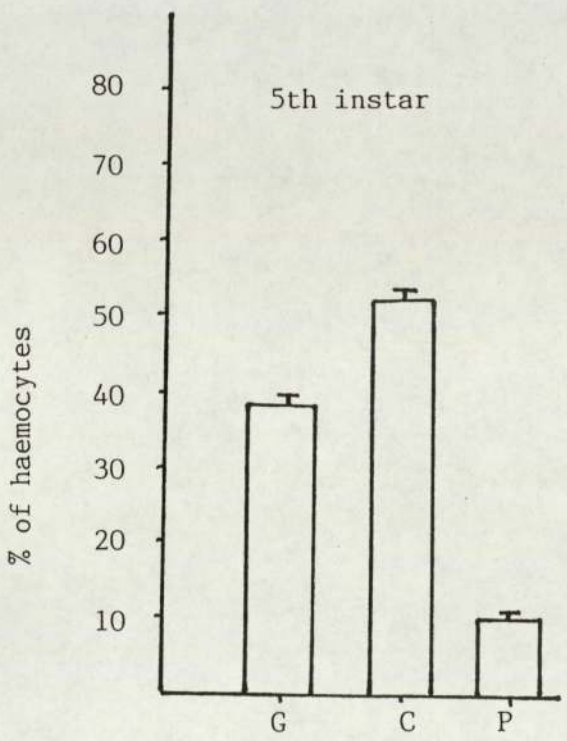
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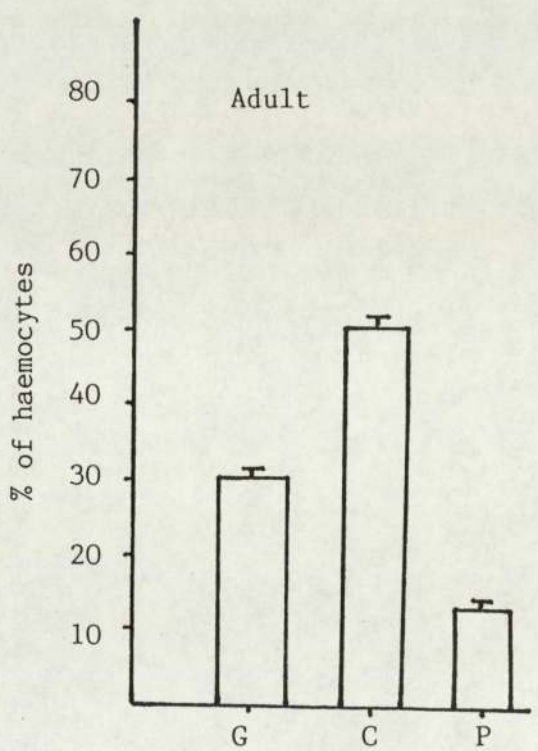
Adult
% of haemocytes
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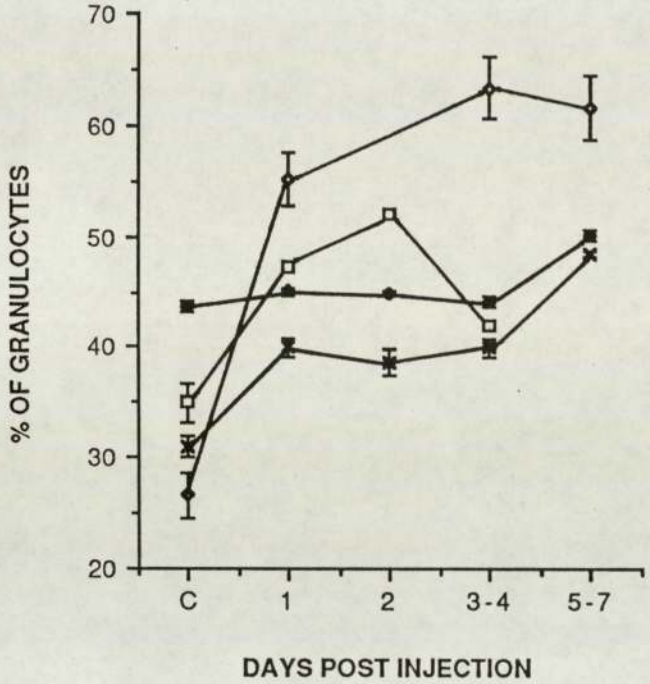
2.3.a



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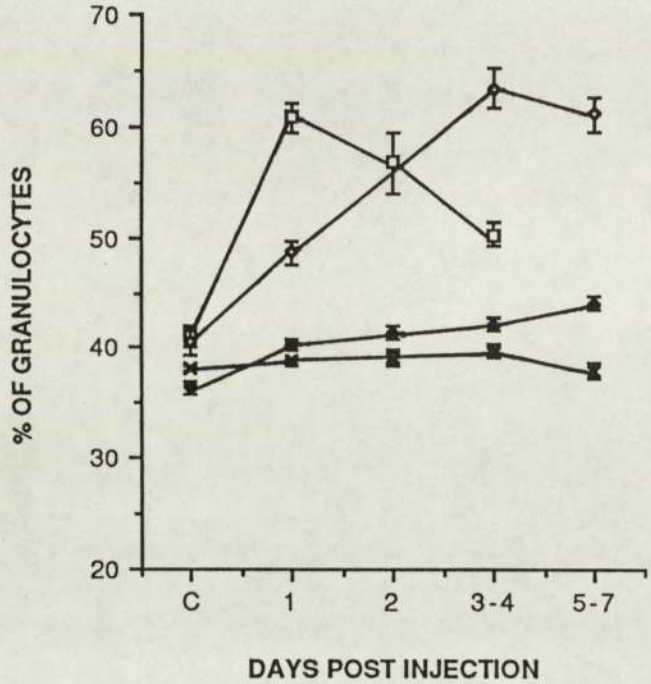

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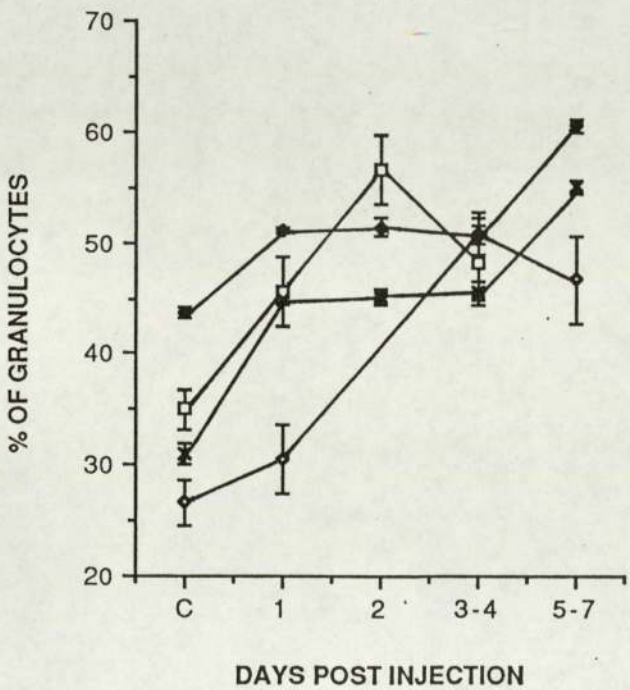

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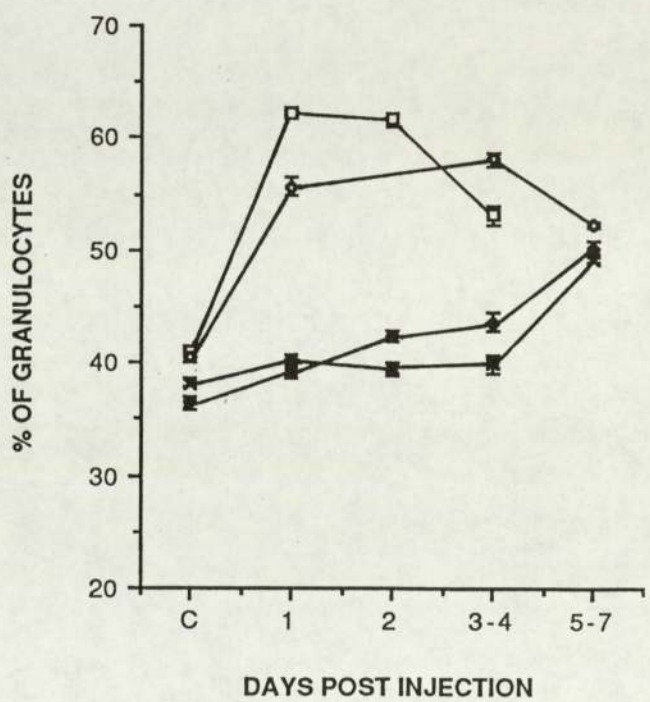

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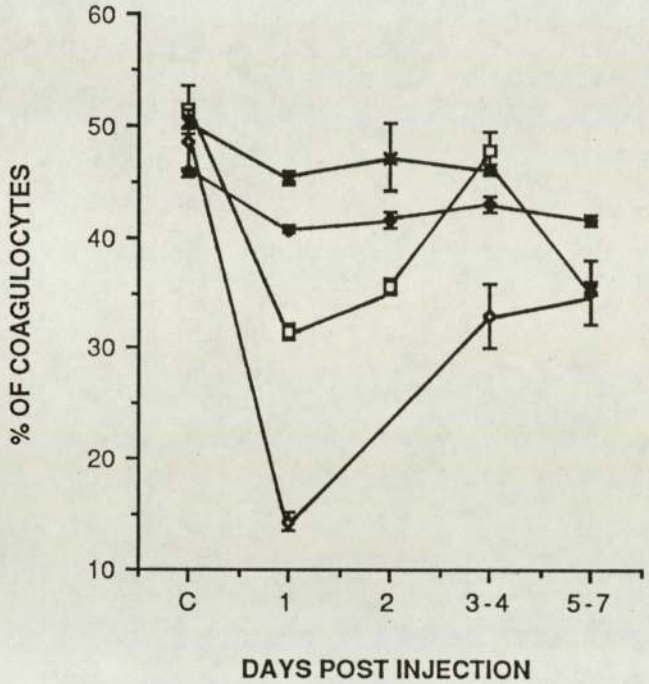

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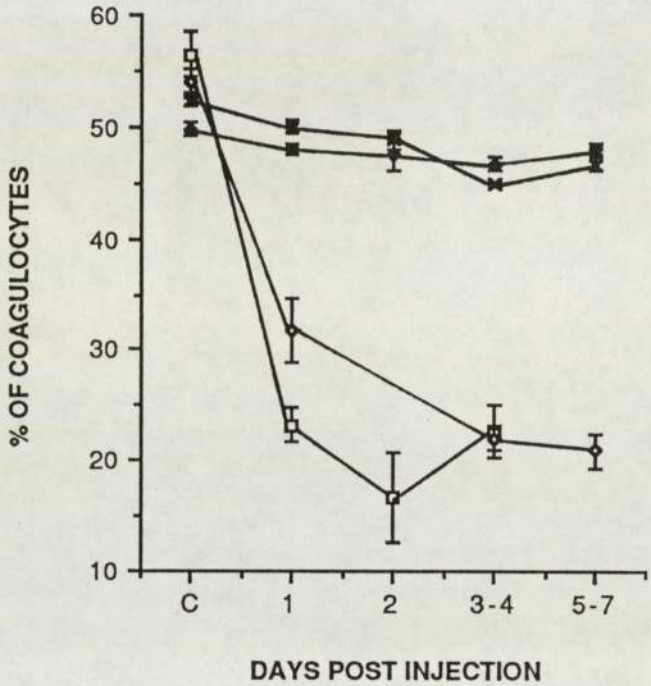

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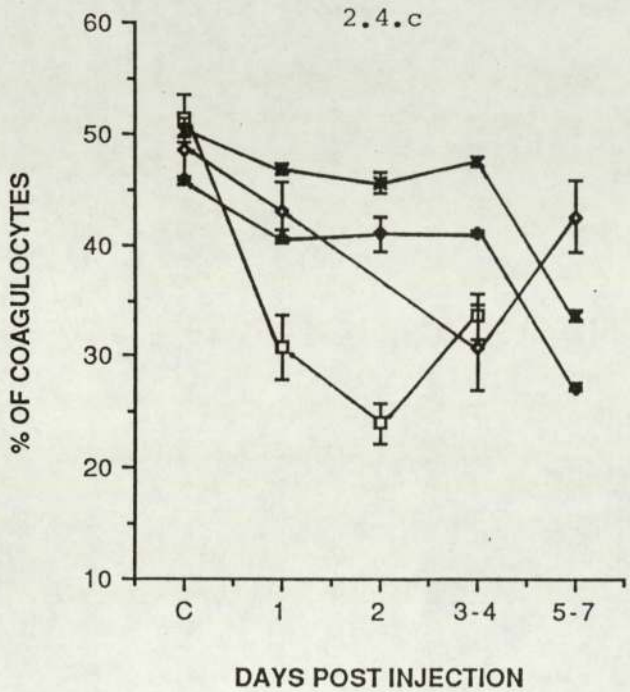

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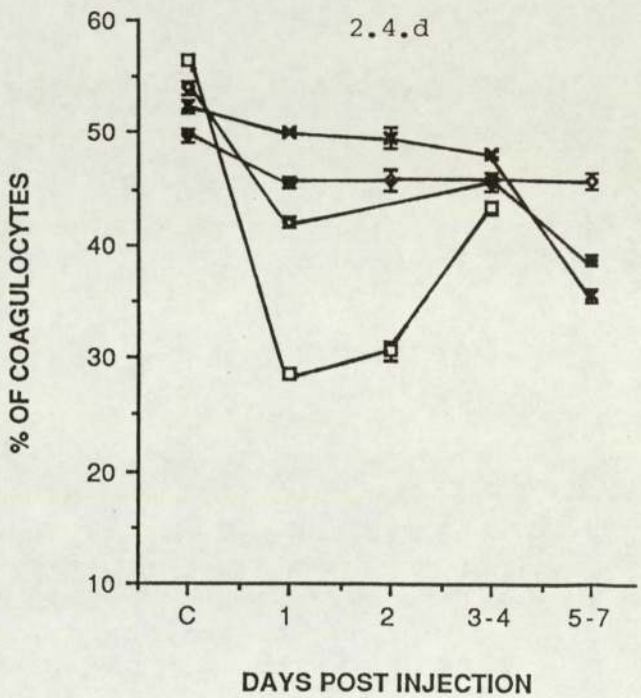

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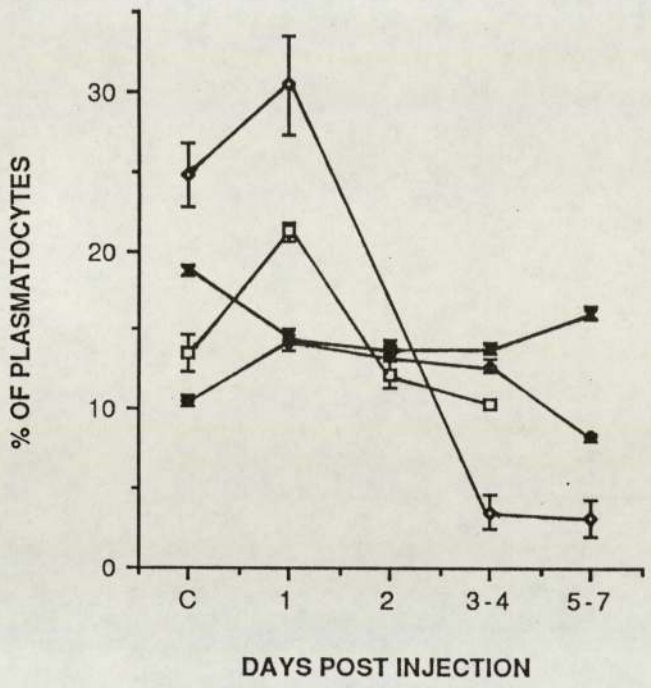

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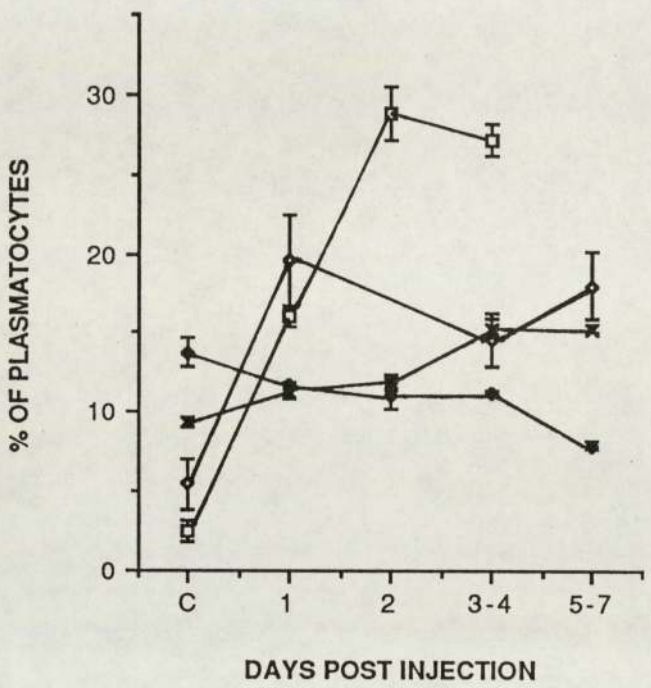

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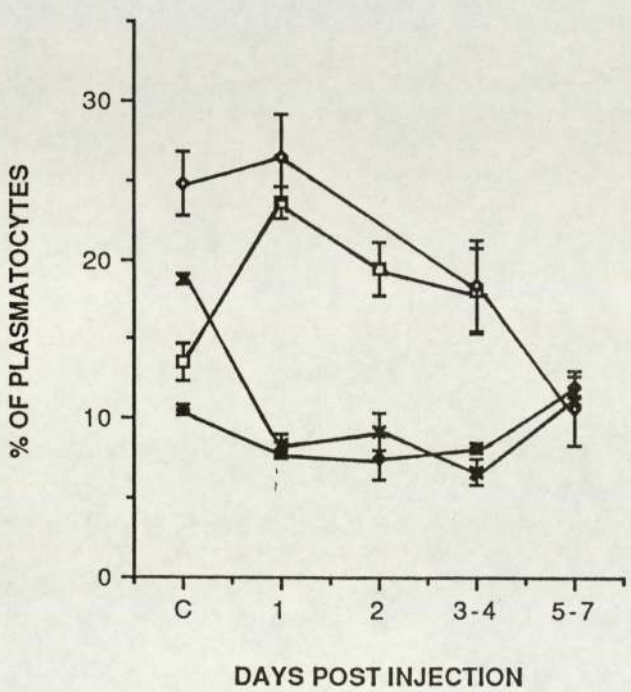

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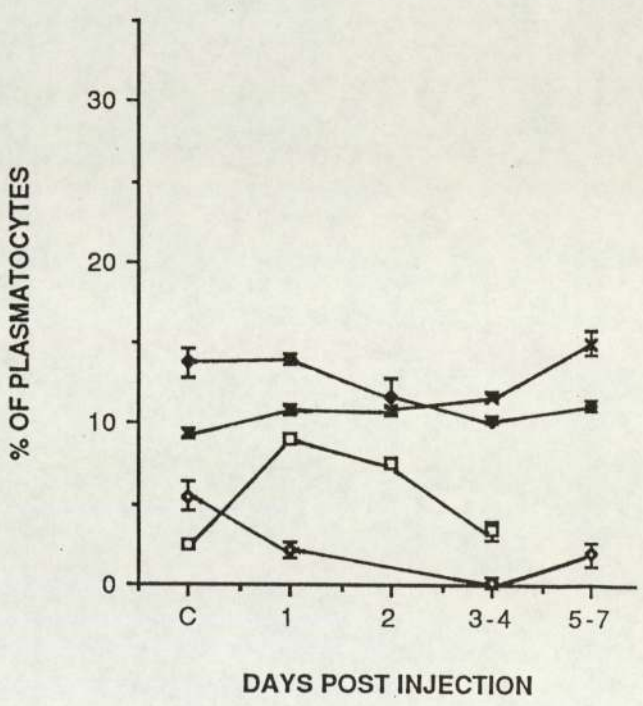

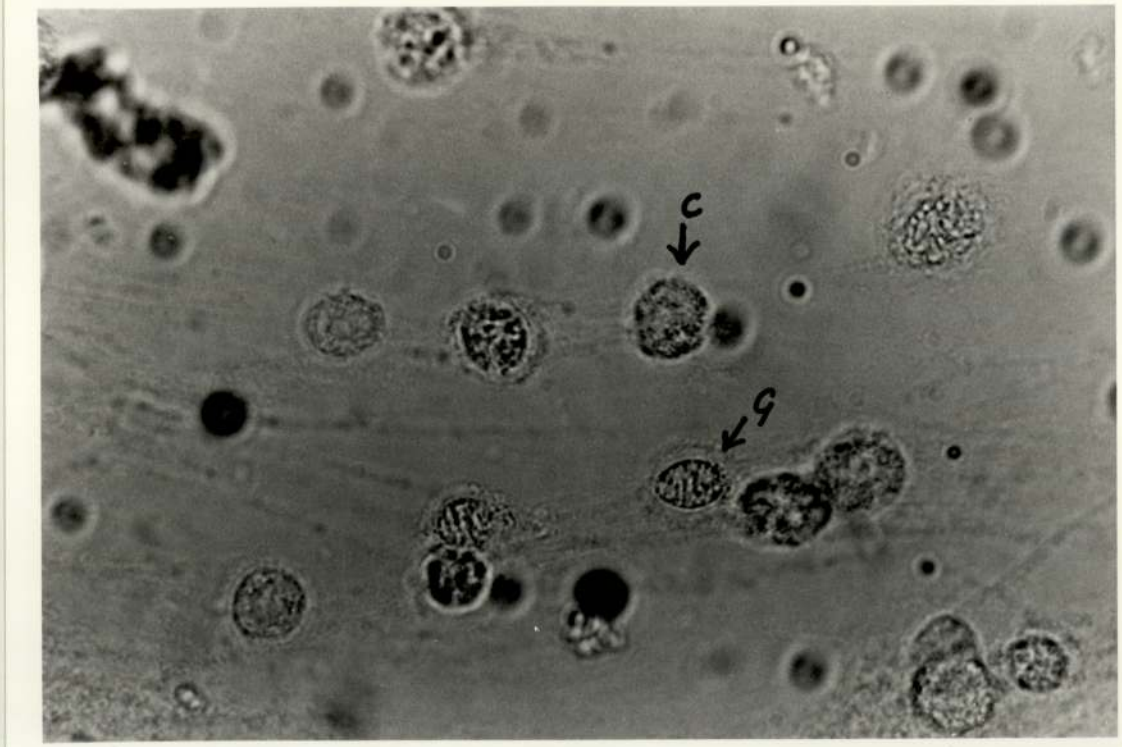
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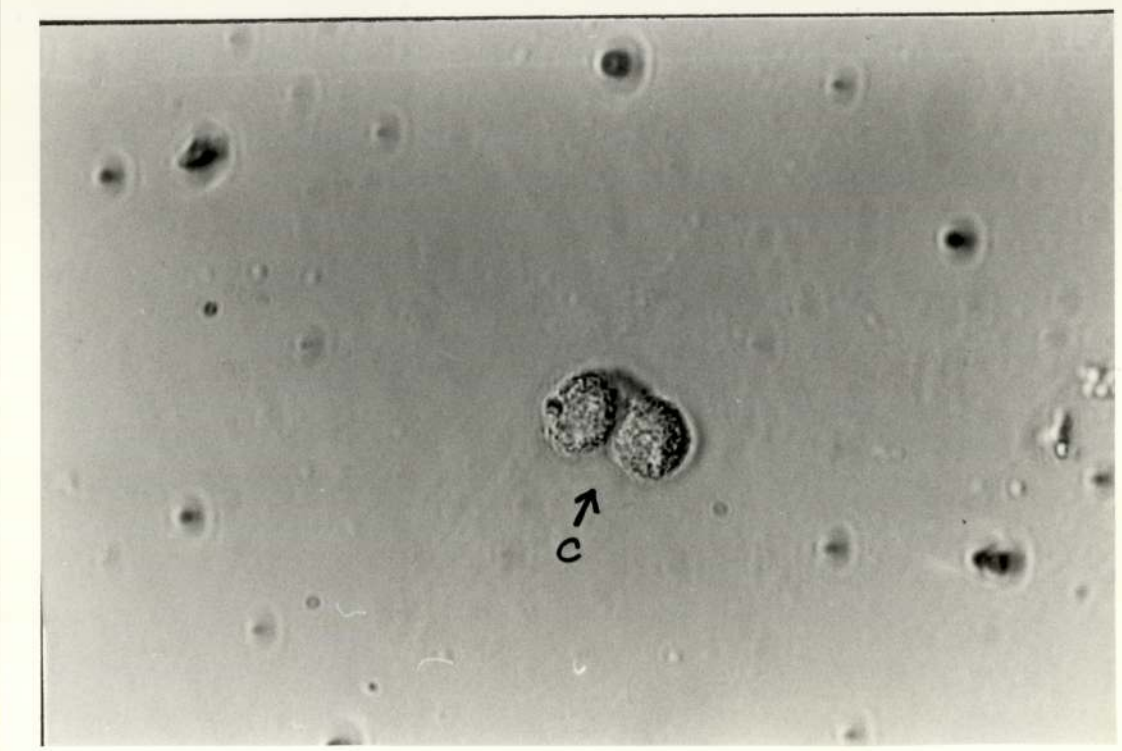
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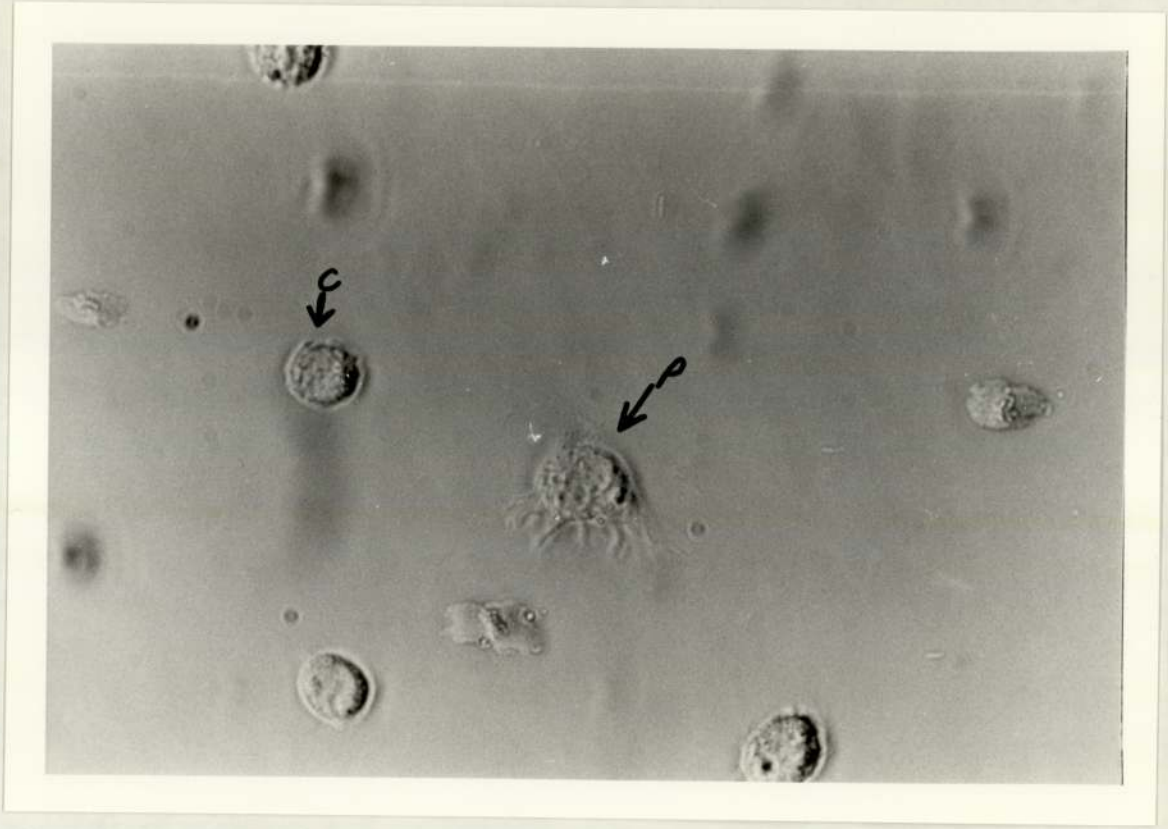
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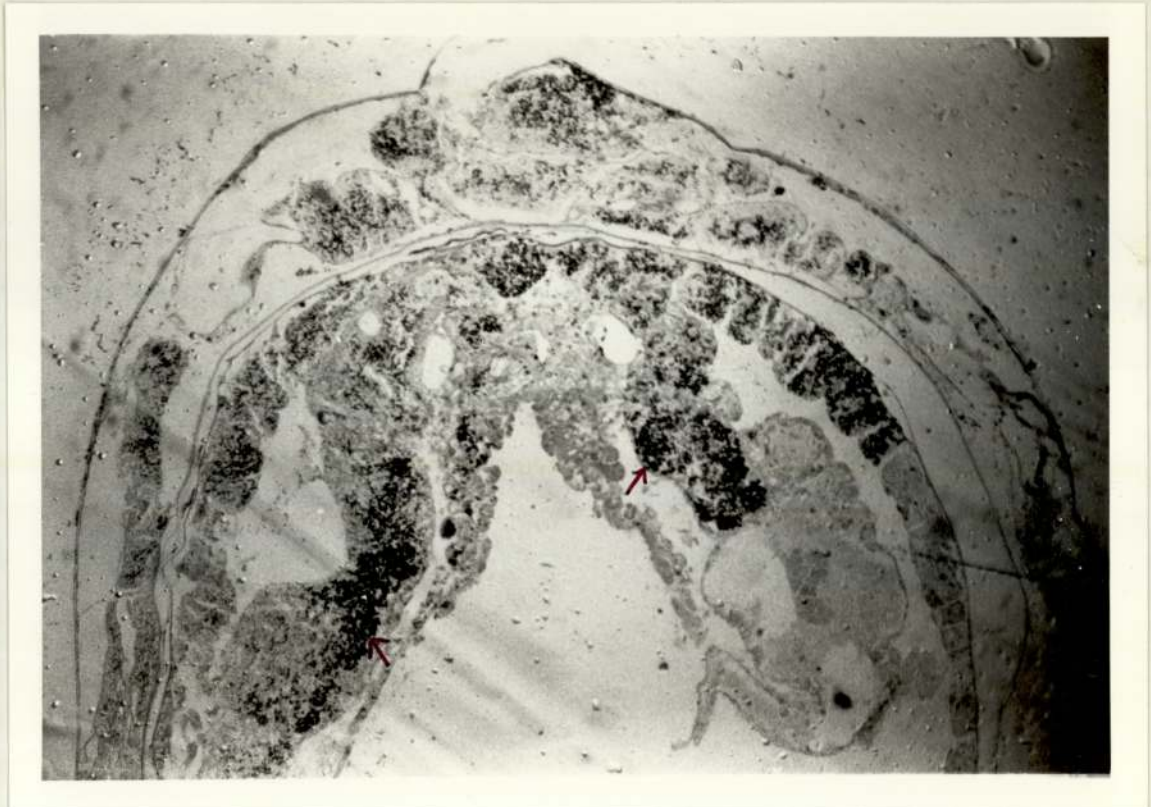


CHAPTER III

THE ONTOGENY AND ACTIVITY OF SERUM AGGLUTININS OF LOCUST

3.1. Introduction

Agglutinins, often referred to as lectins (Ratcliffe, 1986; Renwrantz, 1986; Renwrantz and Stahmer, 1983), are widely distributed in the body fluids of invertebrates, including insects (Ey and Jenkin, 1982) in which they have been reported to be synthesized in the haemocytes (Amirante, 1976) and fat body cells (Komano et al, 1983).

Agglutinins may contribute to a broad self/non-self invertebrate recognition system and thereby augment the neutralization of foreign substances (Olafsen, 1986; Ratcliffe, 1986; Hapner, 1983; Jurenka et al, 1982; Parish, 1977). Barondes (1981) identified lectins as membrane components of various cells. Yeaton (1981) mentioned that there are some discontinuities of occurence of lectins in closely related species, in that some species may have lectins and some do not and individuals at different stages in metamorphosis may have different lectin concentrations or none at all. Several authors report that invertebrate lectins appear to be associated with immunity and defence mechanisms including recognition of foreignness and enhancement of phagocytosis and encapsulation (Cooper, 1974; Marmorosch and Shope, 1975; Whitcomb et al, 1974). Hapner (1983) and Hapner and Jermyn (1981), reported that insect haemagglutinin is a high molecular weight, water-insoluble carbohydrate-binding protein or glycoprotein.

Jurinka et al (1982) found that the haemolymph of Acrididae causes agglutination with human and animal erthrocytes and that haemagglutinin (HA) activity is present in males and females from the 4th instar to the adult stage. Ingram et al (1983) observed that the haemolymph of the American cockroach Periplaneta americana and the desert locust Schistocerca gregaria contains agglutinins against the trypanosomatid flagellates Trypanosoma brucei, Leishmania hertigi and Crithidia fasciculata. Ratcliffe and Rowley (1983) described the (HA) activity in the blood of twenty five species from eleven insect orders. They also carried out immunization and opsonization experiments and studied the ability of insect serum to enhance the recognition and ingestion of test particles. Komano and Natori (1985) mentioned that a humoral lectin plays a role in the defence mechanism of Sarcophaga peregrina. This lectin may cause the lysis of sheep red blood cells introduced into the abdominal cavity of the larvae. Lackie (1981a) investigated the agglutinating activity and the specificity of the serum of locust (Schistocerca gregaria) and American cockroach (Periplaneta americana) by testing the activity against erythrocytes from different species of vertebrates. She found that cockroach serum agglutinated a wider range of erythrocytes than did the locust serum. She also found marked differences between the two species in the range and specificity of the serum agglutinating activity present against erythrocytes, tapeworm larvae (Hymenolepis diminuta), and helminth larvae (Monilifomis dubius) (Lackie, 1976, 1979, 1980a, 1981b; Lackie and Lackie, 1979). Gilliam and Jeter (1970) stated that the adult workers of Apis mellifera produce agglutinating substances after injection with a vaccine prepared from Bacillus larvae.

Many authors have pointed out that there is good evidence that the naturally occuring agglutinin may act as opsonins in invertebrates

(Renwrantz and Cheng, 1977; Renwrantz and Stahmer, 1983; Anderson and Good, 1976; McKay et al, 1969; McKay and Jenkin, 1970). However, other authors have indicated that agglutinins do not act as opsonins (Scott, 1972; Anderson et al, 1972 and 1973a; Rowley and Ratcliffe, 1980; Ratcliffe and Rowley, 1983).

The work presented in this chapter examines the production of agglutinins to a range of different vertebrate erythrocytes and the bacterium E. coli. Studies were carried out on nymphal instars (3rd, 4th and 5th) and adult stages of both Locusta migratoria and Schistocerca gregaria. An important aim of this work was to investigate the ontogeny of serum agglutinin production in locusts. Few systematic investigations of immune ontogeny have been carried out in insects except for a recent study by Rheins and Karp (1985) who observed the ontogeny of the humoral immune response in various developmental stages of the american cockroach, P. americana. Historically, investigators have examined either the immature stages (Briggs, 1958; Stephens, 1962; Chadwick and Vilk, 1969; Boman et al, 1974; Pye and Boman, 1977), or the adult animals (Gingrich, 1964; Seaman and Robert, 1969; and Scott, 1971).

3.2. METHODS AND MATERIALS

3.2.1. Insects and bleeding:

Insects were reared as described in chapter two where the methods of injection and bleeding are also described. The doses of injected antigens for adults and nymphs were also the same as those employed in chapter two.

3.2.2. Antigens:

SRBC in Alsever's solution (Gibco) were washed and prepared as a 1% suspension as described earlier, and chicken red blood cells (CRBC) (Gibco) were prepared by the same method. Mouse red blood cells (MRBC) and rat red blood cells (RRBC) were obtained from animals reared in the laboratory. Mouse and rat blood were collected in heparinised tubes, centrifuged at 1500 rpm and red blood cells were resuspended as a 1% suspension in 0.9% saline solution. E. coli. bacteria were prepared and injected as mentioned in chapter two. Control animals received saline injections only.

3.2.3. Haemolymph collection for haemagglutination (HA) tests:

The nymphal instars and adults of both species of locust were pre-cooled in the fridge at 4°C for 20 minutes to slow down coagulation of the haemolymph and to reduce their activity. They were bled as described previously. The haemolymph was collected into 5 ml. centrifuge tubes kept on ice, and any haemocyte clots were removed by centrifugation at 1500 rpm for 10-15 min. Haemolymph was stored at -20°C when it could not be used immediately (Rowley, 1977a).

3.2.4. Haemagglutinin test procedure:

Microtitrations were performed in the V-shaped wells of disposable haemagglutination trays (Flow) by serially diluting insect haemolymph with 0.9% saline, (25 μl of saline in every well). 25 μl of a 1% erythrocyte suspension was finally added to each well and the trays incubated for one hour at room temperature before examination. HA titres were expressed as the ($-\log_2$) of the last haemolymph dilution that clearly agglutinated the erythrocyte suspensions (Scott, 1972).

3.2.5. Bacterial agglutination:

The procedure for measuring bacterial agglutination was the same as that employed for erythrocytes agglutination but 25 μl of bacterial suspension (9 x 10^5 cells ml) was added to each well instead of the erythrocyte suspensions. The trays were incubated at 28-33°C (the temperature of rearing) for 18 hrs before examination as recommended by Paterson and Firyer (1974).

Agglutinin titres were measured at 1 day, 2 days, 4 days and 7 days post-antigen-injection for adults and 5th nymphal instars; at 1 day, 3 days and 5 days post-injection for 4th nymphal instars and at 1 day, 2 days and 4 days post-injection for 3rd nymphal instars.

3.2.6. The role of haemagglutinins as opsonin-like factors:

In these studies haemolymph from adults and 5th nymphal instars of Locusta migratoria were selected because they contained high HA titres (Table 3.1 - Results) following antigen challenge.

3.2.7. Erythrocyte opsonisation procedure:

The technique employed was based upon that described by Rowley (1977a) and Rowley and Ratcliffe (1980).

3.2.8. Formalised Sheep Erythrocytes: (FSRBC)

FSRBC were prepared by the dropwise addition of 40% formaline (BDH) to a 10% v/v suspension of SRBC in 0.9% saline to give a final concentration of 3% formalin. FSRBC were stored at 4°C and were washed three times with saline before injection as a 1% v/v suspension in saline.

FSRBC were washed 4-5 times in Carlson's saline (Paul, 1975) and after counting in a haemocytometer chamber the concentration was adjusted to 2.4 x 10^7 FSRBC/ml. The suspension was divided into two equal aliquots which were centrifuged at 300g for 5-10 mins. One aliquot was resuspended in an equal volume of haemolymph of a known HA titre (see results) and the other in the same volume of Carlson's saline. Both tubes were then incubated for 45 mins at 26°C on a roller drum apparatus. After haemolymph incubation, any clumps of FSRBC formed were broken into single cell suspensions by ejection several times through a 23 gauge needle attached to 1 ml syringe. The suspensions were checked microscopically to ensure that they contained only single erythrocytes. Saline incubated erythrocytes were similarly treated even though no distinct agglutination had occurred during incubation.

Half of the haemolymph-incubated FSRBC aliquot was then sedimented at 300g for 5 mins and resuspended as a single cell suspension in an equal volume of Carlson's saline. Thus three erythrocyte aliquots were available for incubation with the monolayers: haemoclymph-incubated unwashed; haemolymph-incubated washed; and saline incubated controls.

3.2.9. Haemocyte Collection:

Pre-cooled insects were bled into 12 x 100 mm tubes (contained 1 ml of 5% EDTA as anticoagulant) (Lackie et al, 1985) and the blood was quickly diluted with ice-cold Carlson's saline to give a haemocyte concentration of 5 x 10^5 cells/ml.

3.2.10. Incubation of haemocytes and fSRBC:

0.1 ml of the haemocyte suspension was pipetted onto tissue culture sterile glass coverslips (Miles Laboratories) and left to settle and attach for 15 mins in a petri dish at 26°C. The coverslips were

carefully rinsed with Carlson's saline at least five times with all monolayers to remove non-adherent cells and all traces of haemolymph. Duplicate monolayers from a single insect were overlaid with either:

(a) 0.1 ml of FSRBC in haemolymph which had been incubated in haemolymph and not washed.
(b) FSRBC that had been incubated in haemolymph and washed in Carlson's saline.
(c) FSRBC incubated in Carlson's saline (Control).

A total of at least 6 monolayers was therefore examined from each of 10 insects.

The monolayers were incubated at 26°C in a moist atmosphere for 60 mins, and then vigorously rinsed in Carlson's saline to remove non-phagocytosed FSRBC. Monolayers were fixed in formalin vapour for 10 mins. before examining them with a Zeiss Photomicroscope x 40 phase objective.

To calculate the phagocytic indices (ie. the percentage of haemocytes containing one or more erythrocyte) 250-500 morphologically intact haemocytes were examined in random fields on each monolayer and a mean value calculated from at least two such monolayers. The numbers of phagocytosed FSRBC were also recorded according to the following categories: 1-3; 4-6; 7-9.

Data Analysis:

Levels of significance of the differences between means were determined using Student's 't' test.

3.3. Results

3.3.1. Haemagglutination Responses

The haemolymph of control non-injected and saline injected insects of all the developmental stages examined showed little or no background haemagglutinating activity.

Following injection with foreign erythrocytes, haemagglutination titres in the haemolymph varied in relation to both the antigen used and the developmental stage of the insect concerned. These variations are summarised below and are presented in Figs. 3.1 to 3.3 (and Tables 3.1 to 3.4, Appendix).

(i) Responses in L. migratoria

Following challenge with SRBC young nymphal instars (3rd and 4th) of L. migratoria displayed weak haemagglutinating activity in the haemolymph. Fifth instar nymphs and adult insects both gave much stronger haemagglutination responses against SRBC (Fig. 3.1.a). When challenged with MRBC, however, the younger stages (3rd, 4th and 5th instars) all responded by producing stronger agglutinin titres than the adult insects (Fig. 3.2.a). Both RRBC and CRBC appeared to be poorly immunogenic for all developmental stages of L. migratoria, with the exception of fifth nymphal instars which showed a weak agglutinin response towards RRBC (Table 3.3, Appendix).

(ii) Responses in S. gregaria

Responses to SRBC by S. gregaria appeared to be weaker than those observed in L. migratoria. No haemagglutinins were detected in the third instar nymphs amd weak reactions were observed in fourth and fifth instars (Fig. 3.1.b). Adult insects failed to produce significant increases in haemagglutination titres following SRBC challenge. When challenged with MRBC, third and fourth instar nymphs gave weak agglutinin responses, but adults and fifth instar nymphs of S. gregaria failed to respond to this antigen (Fig. 3.2.b). As with L. migratoria, haemagglutination responses to RRBC and CRBC were absent in all stages tested, the only exception being a weak response of fifth instar nymphs towards RRBC (Table 3.3, Appendix).

3.3.2. Bacterial agglutinins

(i) Responses in L. migratoria

The responses of L. migratoria to E. coli are shown in Table 3.4. Following challenge, third instar nymphs failed to respond to E. coli, but there was a slight increase in bacterial agglutination titres in the haemolymph of fourth instar nymphs on the third day post-injection. Fifth instar nymphs responded much more strongly to bacterial challenge and although adult insects also produced bacterial agglutinin titres, these were less than those observed for fifth instars (Fig. 3.3.a).

(ii) Responses in S. gregaria

Both third and fourth instar nymphs failed to respond following challenge with E. coli (Fig. 3.3.b). The responses of adults and fifth instar nymphs were weak, but low agglutinin levels were detected at one

day post-injection and persisted for seven days when observations ceased.

3.3.3. Opsonization experiments with haemagglutinins

The results shown in Table 3.5 demonstrate that pre-incubation of FSRBC with haemolymph from a pre-immunised animal results in an increased uptake of erythrocytes by phagocytic haemocytes in culture. This was true of both "pre-incubated" and "pre-incubated and washed" FSRBC. Since the phagocytic indices for both categories of erythrocytes exposed to immune haemolymphs were higher than for FSRBC incubated with saline alone. The results indicate that factor(s) in the haemolymph of SRBC immunised locusts (*L. migratoria* are capable of promoting phagocytosis of FSRBC *in vitro*.

3.4. DISCUSSION

The experiments reported here have examined the ontogenesis of inducible immune responses towards foreign erythrocytes and the bacteria *E. coli*.

The haemagglutination experiments revealed that low background levels of agglutinating activity were sometimes present in *L. migratoria* but such activity was often lower in *S. gregaria*. The present findings contrast with those of Rowley (1977a) who stated that, in general, haemagglutinating activity was absent in orthopterans, and Jurinca et al. (1982) who reported that *S. gregaria* haemolymph showed no haemagglutinating activity. Present attempts to enhance background levels of agglutinating activity by antigen injection gave variable results depending upon the antigen used and the age of the insects at the time of injection. Both CRBC and RRBC appeared to be weak antigens

compared with SRBC and MRBC. In general, the younger stages (3rd and 4th instars) of locust produced little or no response to foreign erythrocytes, the exception being after injection with MRBC, Interestingly, the latter antigen elicited relatively poor responses in adult locusts. The reasons for this apparent anomaly are not known, but the findings may reflect the emergence and subsequent disappearance of antigen receptors at different stages of ontogeny. The negative or low haemagglutinating activity of younger nymphs to some erythrocyte antigens employed here may be due to the nymphs'inability to process the antigen as effectively as more mature stages (see phagocytic studies in Chapter Four). However, the cell profile (see Chapter Two) of third and fourth nymphal instars is similar to that seen in adults and fifth instar stages, and they are certainly able to respond to antigens such as MRBC. A possible explanation for the poor responsiveness to certain antigens may be that the young nymph has either fewer cells with appropriate receptors for those antigens or a preponderance of low affinity receptors when compared to fifth instars and adults.

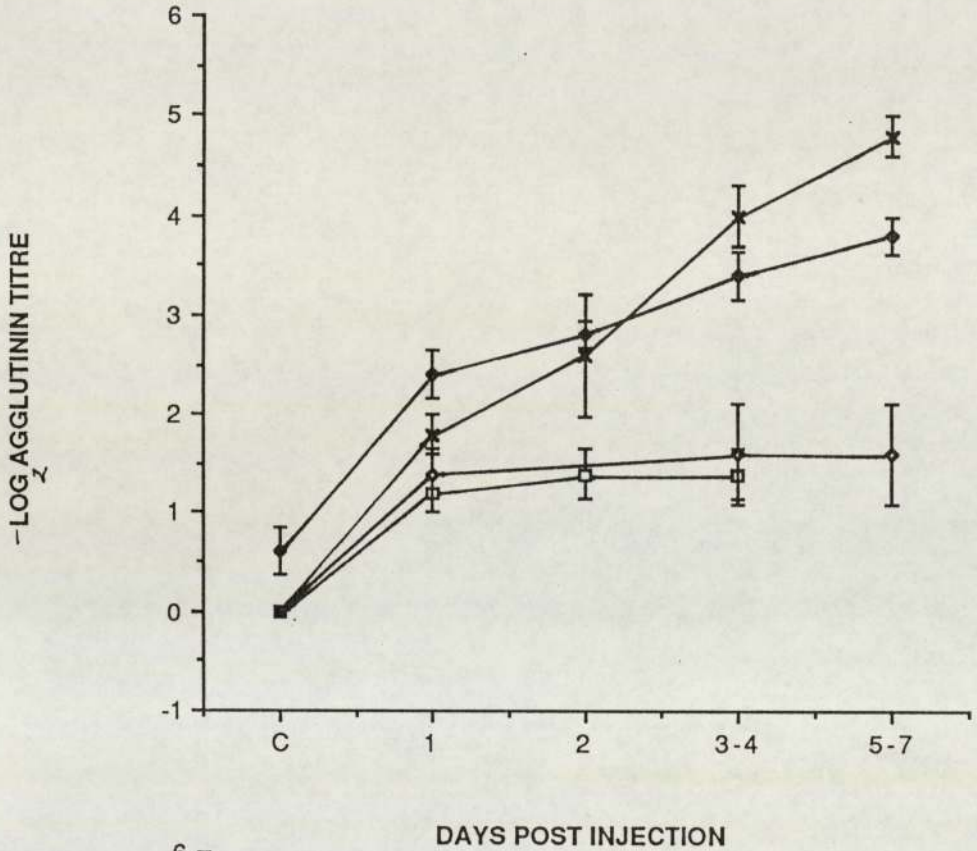
Where haemagglutinins were produced at higher titres, it was possible to examine their specificity in cross-reactivity tests. These tests revealed that the agglutinins appeared to be specific for the inducing agent, although it must be borne in mind that no agglutination of CRBC occurred even when animals were immunised with this antigen. The recognition processes involved in agglutination by insect lectins are not well understood. Most studies have been carried out on crude haemolymph and relatively few studies have utilised purified fractions with agglutinating activity. Olafsen (1986) and Renwrantz (1986) have recently reviewed the occurrence and functions of invertebrate lectins. Lectins are generally defined as carbohydrate binding, non-immunoglobulin proteins that agglutinate cells through interaction with

glycoproteins or glycolipids (Boyd and Shapleigh, 1954) and have consequently been suggested to play a role in immunological defence systems as molecules which may cause clumping of viruses, bacteria, cellular microinvaders, and their secreted products. Renwrantz (1986) has pointed out that unless microorganisms are present at high densities in the haemolymph it is unlikely (under natural conditions) that they will reach a concentration where agglutination is possible. A more likely function, he proposes, is that lectins such as agglutinins may promote agglutination between foreign microorganisms and haemocytes of the host. If lectin-induced binding of foreign particles to the surface of haemocytes promotes ingestion then lectins may function as opsonins. Opsonising properties of invertebrate lectins have been reported by a number of authors (Anderson and Good, 1976; McKay and Jenkin, 1970; Pauley, 1974; Pauley et al., 1971; Ratcliffe and Rowley, 1984; Robohm, 1984; Tripp, 1966). This is borne out to some extent by the studies reported here where exposure of FSRBC to haemolymph taken from immunised animals, greatly increased the rate at which these cells were phagocytosed by haemocytes in culture. Increased phagocytosis was seen even when erythrocytes which had been pre-incubated with immune haemolymph were washed prior to culture, suggesting perhaps that the opsonising factor(s) binds fairly tightly to the surface of the foreign cell. The experiments reported here only utilised crude haemolymph and it remains to be determined whether the same or different fractions are involved in both haemagglutination and opsonisation. It should also be mentioned that in addition to lectins in the haemolymph and body fluids of invertebrates, evidence has recently emerged for membrane bound lectins on the surface of snail (Lymnaea stagnalis) haemocytes (Van der Knaap et al., 1981). Antibodies raised against serum lectin were also found to cross-react with the surface membrane of haemocytes. Whether

the lectin is synthesised by haemocytes or passively acquired from the serum is not known.

The function of insect agglutinins *in vivo* remains unclear. It would be of interest, therefore, to investigate whether passive transfer of haemolymph from immunised locusts to controls had any effect on the clearance rates of injected antigens such as SRBC or bacteria.

Owing to limitations of time and space, the experiments reported here were concluded after seven days of observation when, in many cases, the agglutinin titres were still high. Further studies are, therefore, planned to monitor the persistence of this heightened reactivity. Another aspect of immunological reactivity which was not studied here is memory. It remains to be seen whether subsequent doses of a sensitising antigen will result in a more rapid and/or a heightened level of reactivity. Conversely, it would be interesting to study whether early exposure of immature stages to antigens might result in tolerance.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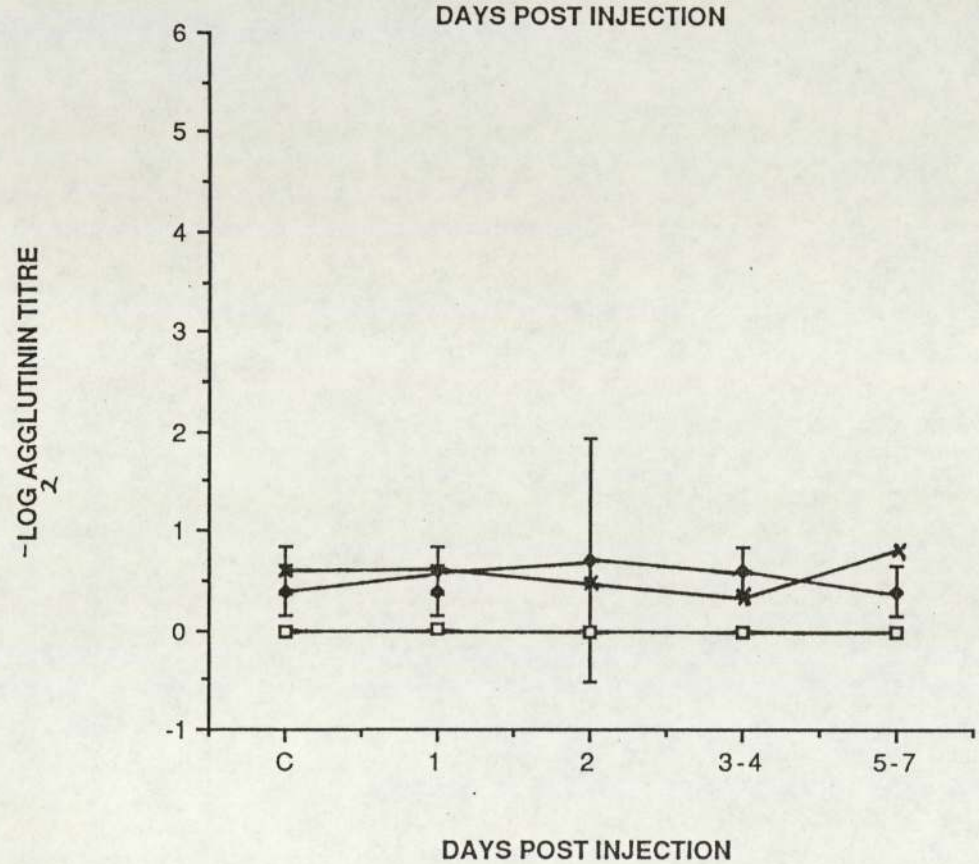
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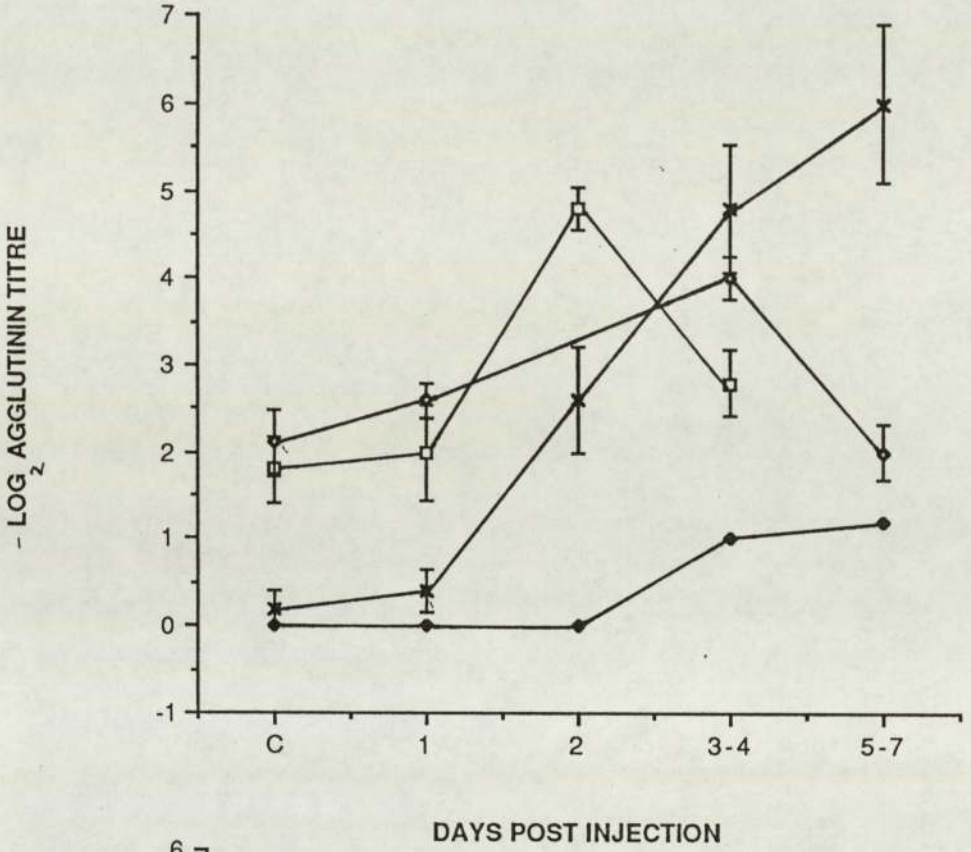
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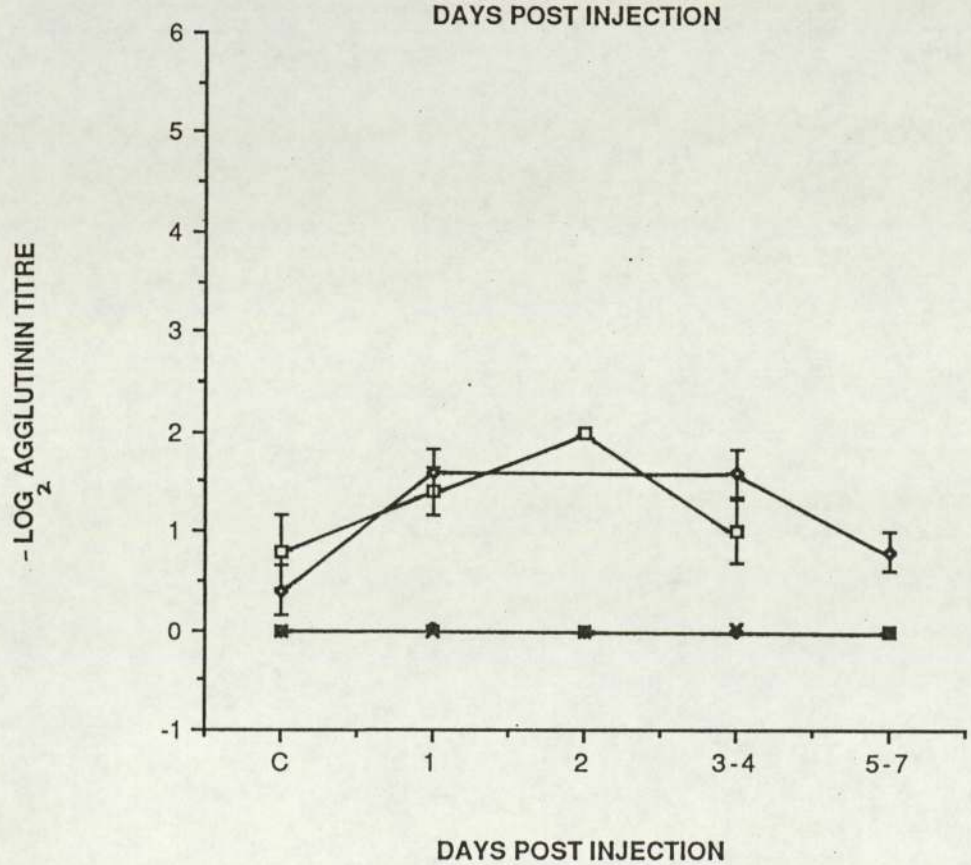
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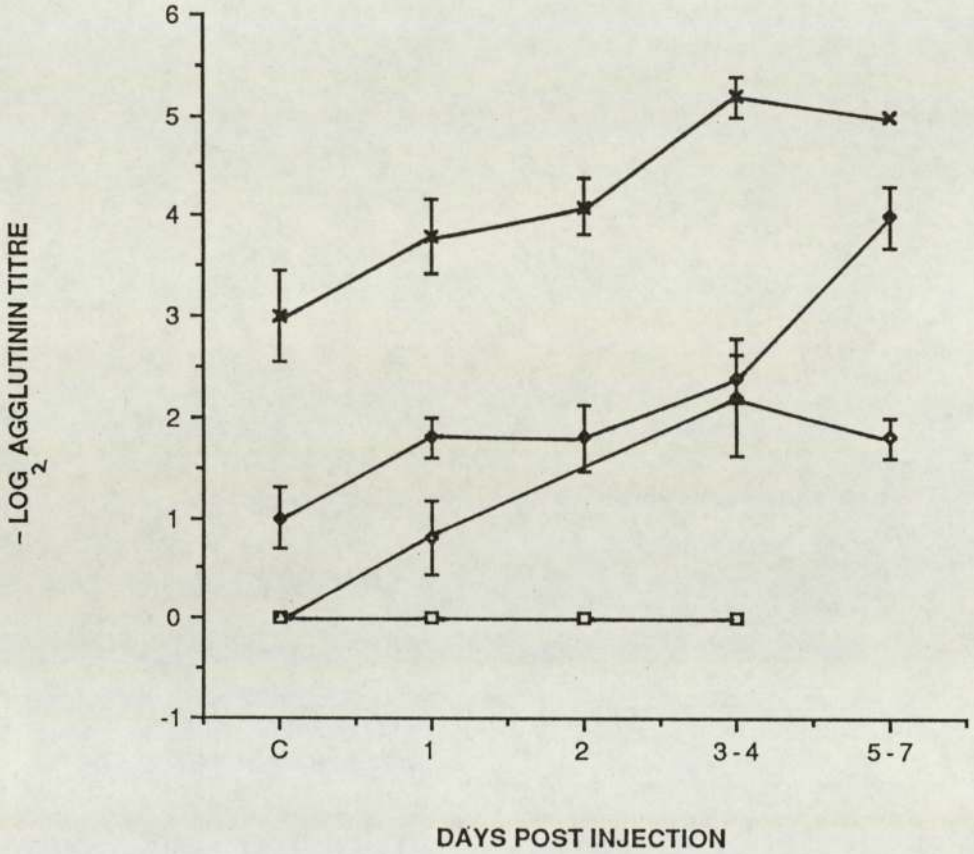
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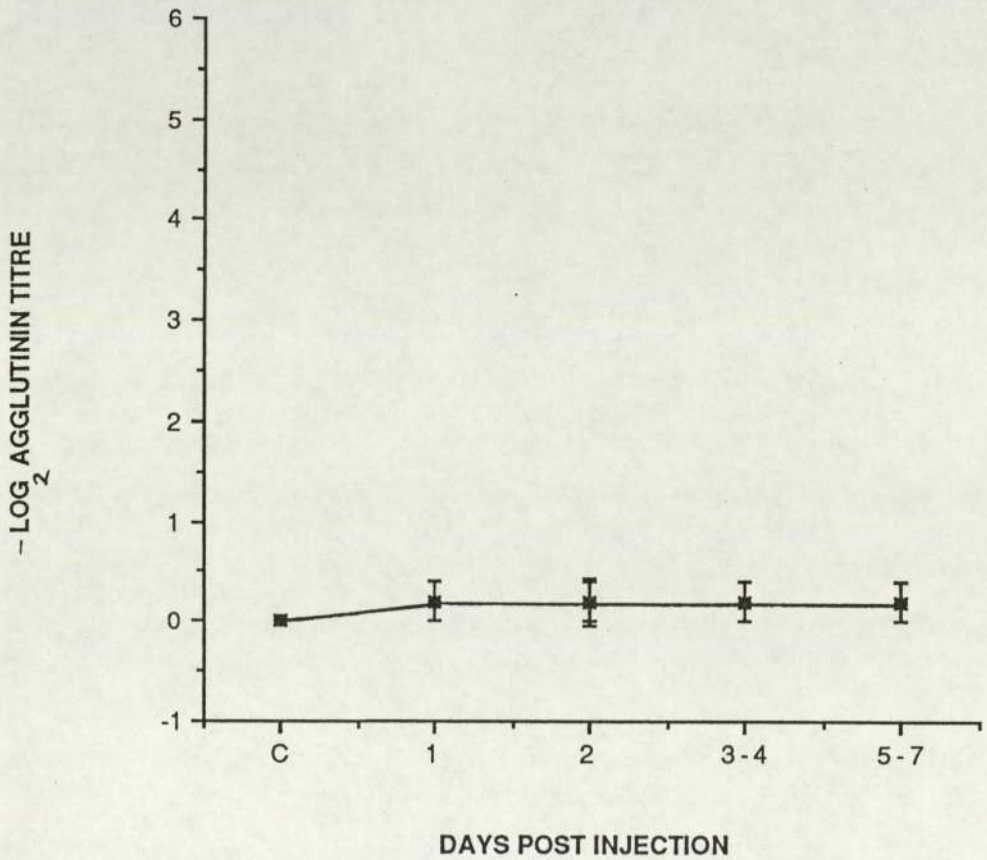
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CHAPTER IV

THE ONTOGENY OF PHAGOCYTIC ACTIVITY IN HAEMOCYTES AND HAEMOPOIETIC TISSUE

4.1 Introduction

Phagocytosis is the process whereby a cell engulfs particles by drawing them into its cytoplasm. Since Elie Metchnikoff's (1884) pioneering studies at the turn of the century, many authors have described the phagocytic process in a wide range of vertebrates and invertebrates. Metchnikoff proposed that cells capable of phagocytosis and digestion of foreign microorganisms played a major role in protecting animals against infection, and research on phagocytosis in insects has revealed that insect haemocytes are indeed capable of ingesting viruses, bacteria, protozoans and fungi both *in vivo* and *in vitro* (Jones, 1962; Salt 1970; Whitcomb et al., 1974). Once the physical and chemical barriers provided by the gut and cuticle have been breached, invading pathogens may enter the haemocoel and these become exposed to the circulating haemocytes. Under these circumstances, phagocytosis represents the primary cellular defense reaction in the haemolymph. Thus, in the waxmoth, *Galleria mellonella*, phagocytic haemocytes are capable of effectively removing bacteria from the haemolymph at concentrations upto 10 microorganisms haemolymph (Ratcliffe and Walters, 1983). Kaaya et al. (1985) described the phagocytosis of bacteria injected into the haemocoel of the fly, *Glossina morsitans*, and bacterial phagocytosis *in vitro* has been demonstrated by Shozawa et al. (1985) in haemocytes of *Mamestra brassicae*. Studies on the phagocytosis of different types and strains of bacteria by different

species from different orders of insects have been carried out by many authors (Anderson et al., 1973; Ratcliffe and Rowley, 1974; Tyson and Jenkin, 1974; Gagen and Ratcliffe, 1976; Rowley and Ratcliffe 1976 a,b,c; Rowley, 1977a; Brehelin, Zachary and Hoffmann, 1978; Walters and Ratcliffe , 1980).

Whilst much of the research on phagocytosis in insects has focussed upon bacterial uptake, more recently a number of workers have studied the phagocytosis of protozoans by insect haemocytes. Takle (1975) found that the clearance of Trypanosoma rangeii from the haemocoel of Schistocerca gregaria and Periplaneta americana involved both phagocytosis and nodule formation. Studies on insect vectors of protozoan diseases have also demonstrated that haemocytes of the vector species are able to ingest invating protozoan pathogens. Thus, phagocytosis of several species of flagellate has been reported in Rhodnius prolixus (Tobie 1968; Molyneux et al., 1986), and similar findings have been reported for haemocytes of Culex pipiens and Aedes aegypti which are able to phagocytose Plasmodium gallinaceum (Weathersby and McCall, 1968).

Studies of phagocytosis in insects have revealed that plasmatocytes are the major phagocytic cells and that granular cells and cystocytes may also be involved (Neuwirth, 1973; Brehelin et al., 1978). In addition to phagocytosis by freely circulating haemocytes, many insects trap particulate material in specialised haemopoietic or phagocytic organs (Jones, 1970). In Locusta migratoria, for instance, there is an irregular accumulation of cells on the upper surface of the dorsal diaphragm which extends along the length of the dorsal vessel. Several cell types are

present in such dorsal accumulations including reticular cells; immature, differentiating haemocytes; and fully differentiated cells of the different cell categories normally found in the circulation (Salt, 1970; Hoffmann, 1970, 1973, 1980; Hoffmann et al., 1968b; Hoffman et al., 1979; Hoffmann and Porte, 1973). Studies have shown that this 'dorsal haemopoietic tissue' is involved in the intracellular trapping of injected inert particles or microorganisms from the circulation (Hoffmann et al., 1974). The reticular cells found in the haemopoietic organs of L. migratoria do not enter the circulation and are thought to be similar to the reticular cells of vertebrate lymphatic tissue (Ratcliffe, 1986). They may also release antibacterial factors into the blood (Brehelin and Hoffmann, 1980).

Previous studies have shown that the rate of phagocytic ingestion in insects is influenced not only by the nature of the foreign material, but is also affected by environmental factors such as temperature, season and pH (Whitcomb et al., 1974). To date, little attention has been paid to the ontogeny of phagocytic responses in insects, and the main aim of the present chapter is to investigate the phagocytosis of non-biotic (charcoal and indian ink) and biotic (SRBC) materials by haemocytes of different developmental stages and adults of Locusta migratoria and Schistocerca gregaria. Phagocytic haemocytes were examined by phase contrast microscopy following exposure to foreign particles in vivo. Histological studies have also been employed in order to reveal major sites of phagocytosis in both species of insect.

4.2 Materials and Methods

4.2.A Injection of particulate material

Nymphal instars and adults of both species of locust were maintained and injected as described in chapter two.

For fifth instars and adult locusts the injection volume was 10 µl and three types of foreign particle were employed. Charcoal was injected in the form of a 0.5% v/v suspension in 0.5% saline. Indian ink (Pelikan) was administered as a dilution of one part ink to five parts 0.5% saline solution, and SRBC (1%) were injected as described earlier.

For third and fourth nymphal instars mortality was high following injection and so the concentrations of particulate material injected were adjusted accordingly. The volume injected was also reduced to 2 µl. Charcoal was injected as a 0.1% suspension and Indian ink (which was found to be particularly toxic to younger stages) was given in the form of a 1:20 dilution in saline. Sheep erythrocytes were administered as a 1% suspension.

4.2.B Estimation of phagocytosis

Phagocytosis in haemocytes and haemopoietic tissue was examined at intervals post-injection. For adult and fifth instar locusts groups of five animals were sampled at each time point post-injection. One drop of haemolymph was collected from each animal and prepared for phase contrast microscopy as previously described. For each sample, 100 cells were examined and the percentage of haemocytes containing phagocytosed particles was calculated. One extra sample, taken at random from one of the five insects examined,

was also counted. For third and fourth instars it was necessary to pool haemolymph from 10-15 insects. One drop of pooled haemolymph was placed on each of six slides and phagocytosis estimated as described above.

Uptake of foreign particles by the haemopoietic tissue was examined histologically by light microscope. Tissues were fixed overnight at room temperature in 10% formaldehyde prior to wax embedding. Five micron sections were cut and stained with Harris's haemotoxylin and eosin after the method of Hoffmann (1973) modified by Hoffmann et al. (1974). These studies utilised charcoal and Indian ink particles only, since it was felt that these particles would be more persistent in vivo and thus more easily detected in histological sections than SRBC.

4.3 Results

4.3.A Phagocytosis by haemocytes

The present study revealed that the plasmatocytes and coagulocytes of both Locusta and Schistocerca play an important role in the phagocytosis of foreign particles. Both cell types ingested charcoal, Indian ink and SRBC and examples of phagocytic cells are shown in Figs. 4.7 to 4.9.

The raw data on phagocytosis of charcoal, indian ink and SRBC are presented in Tables 4.1 to 4.3 (Appendix). This data forms the basis of Figures 4.1 to 4.6 which summarise the percentages of phagocytic haemocytes in the haemolymph of both locust species at different developmental stages and at different times post-injection. Figs. 4.1 to 4.4 reveal that, with one exception,

haemocytes of third and fourth nymphal instars failed to phagocytose charcoal and Indian ink particles when examined from 1-5 days post injection. Haemocytes of third instar Locusta migratoria demonstrated low (6%), transient levels of phagocytosis of Indian ink particles at one day post-injection, but this was not apparent at later time intervals.

In contrast, haemocytes of fifth instar nymphs and adults of both species were able to phagocytose charcoal and Indian ink particles. Small numbers of cells containing phagocytosed particles were detected as early as one day post-injection and these increased and persisted in the haemolymph until observations ceased 5-7 days after challenge.

Despite their poor response to non-biotic particles haemocytes of both third and fourth instars were able to phagocytose SRBC in considerable numbers. This is illustrated in figures 4.5 and 4.6 where it may be seen that, even at one day post-injection, from 15-18% of haemocytes in both locust species had ingested foreign erythrocytes. These figures declined thereafter but small numbers of phagocytic cells were still present in the haemolymph almost a week later.

Phagocytosis of SRBC by haemocytes of fifth instar nymphs was about the same as that observed in the younger stages, but the percentage of cells exhibiting phagocytosis was more persistent and remained fairly constant until observations ceased 5-7 days post-injection. The response of adult haemocytes to SRBC was very strong. In Schistocerca almost 85% of all haemocytes observed at

one day post-injection contained one or more sheep erythrocytes. The figure for Locusta haemocytes was lower but still reached 65% two days after challenge. The peak phagocytic response for both species of locust was observed during the first two days post-injection, but approximately 25% of all adult haemocytes still contained phagocytosed erythrocytes one week after the start of the experiment.

4.3.B Phagocytosis by haemopoietic tissue

Inspection of histological sections of haemopoietic tissue taken from injected third and fourth instars revealed no evidence of phagocytosed particles. In both fifth instar nymphs and adult locusts of both species, injected foreign particles were taken up by the reticular cells of the haemopoietic tissue and also by the pericardial cells. Trapped particles were observed from 1-day post-injection. Figure 4.10 illustrates the cardiac vessel and pericardial cells of control, non-injected, adult locust. This may be compared with Figs. 4.11 and 4.12 which depict the phagocytosis of Indian ink particles and charcoal particles in the haemopoietic tissue of L. migratoria four days post-injection. In some slides the haemopoietic tissue of injected locusts showed signs of hypertrophy.

4.4 Discussion

The present study has shown that the plasmatocytes and coagulocytes of both L. migratoria and S. gregaria play an important role in the insects' defence reactions against injected particulate material. Both cell types were observed to ingest charcoal, Indian ink and SRBC. These findings are in agreement with those of

Brehelin and Hoffmann (1980) who also found that the uptake of injected material in L. migratoria was carried out by plasmatocytes and coagulocytes which were seen to form "rosettes" with the injected particles prior to engulfing them.

Phagocytosis by insect haemocytes is influenced not only by environmental factors such as temperature but also by the nature of the foreign material itself (Whitcomb et al. 1974; Ratcliffe, 1986). This was clearly demonstrated in the present study where biotic particles (SRBC) initiated a much stronger phagocytic response in both adult and juvenile locusts than non-biotic particles (charcoal and Indian ink). The factors governing the rate at which different foreign particles are ingested by insect phagocytes are not fully understood, but a number of recent papers have begun to investigate this issue. For instance, haemocytes of G. mellonella larvae do not normally phagocytose the bacterium Bacillus thuringiensis subtoxicus. However, if primed with an injection of latex beads they will do so. This phagocytic activity can be conferred upon naive larvae by the transfer of cell free haemolymph from latex primed individuals, thereby suggesting the production of a soluble stimulatory factor (or factors) which can promote phagocytosis (Mohring et al., 1979 a,b). A similar factor has been reported in the haemolymph of P. americana which increases the rate of ingestion of heat killed bacteria by haemocytes from the same species (Ratcliffe and Rowley, 1983). The nature of these stimulatory factors is not known, but Leonard et al. (1985) demonstrated that activation of the prophenoloxidase system by microbial extracts significantly enhances the phagocytic uptake of particles in insect

haemocyte monolayer cultures. Thus, laminarin (a component of fungal cell walls) and bacterial lipopolysaccharide both promote a significant increase in the phagocytosis of heat-killed bacteria in vitro when compared to dextran and saline treated controls. The addition of a serine protease inhibitor (p-nitrophenyl-p-guanobenzoate) removed the stimulatory effect of laminarin and, since proteases are involved in the activation of the prophenoloxidase system, this has been taken as evidence for the enhancement of phagocytosis by increased prophenoloxidase activity. This view is supported by the finding that insect haemocytes exposed to laminarein in vitro showed increased levels of phenoloxydase activity in their cytoplasm. These studies have recently been reviewed by Ratcliffe (1986) who also suggests that laminarin may promote phagocytosis by acting as a bridging molecule between agglutinin coated particles and the haemocyte surface. The relatively poor response to non-biotic particles observed in the present study may be related to the relative inefficiency of charcoal and Indian ink particles in stimulating prophenoloxidase activation. Further studies of the kind described by Ratcliffe (1986) are required to clarify this issue.

An alternative or additional mechanism promoting phagocytosis by insect haemocytes may involve agglutinins (see chapter three). Only recently has evidence for the role of invertebrate agglutinins in phagocytosis been provided. Renwrantz and Stahmer (1983) were able to demonstrate that purified aggutinin of the molluse Mytilus edulis acted as an opsonin in vitro, and increased the percentage of cells phagocytosing yeast cells from 5% in control haemocytes, to 55% in

those exposed to the purified agglutinin molecule. Opsonic factors have also recently been described in the haemolymph of crustaceans (Smith and Soderhall, 1986). Definitive studies on the role of insect agglutinins in phagocytosis are still lacking, but agglutinins have been detected on the surface of insect plasmatocytes and granulocytes but are absent on non-phagocytic cells (Yeaton 1980; Komano et al. 1983). The results presented in chapter three of this thesis have shown that agglutinins are produced soon after SRBC injection in both species of locust and this may, in part, account for the more efficient uptake of foreign erythrocytes when compared to charcoal and Indian ink. Inert particles which do not stimulate agglutinin production may be phagocytosed less readily.

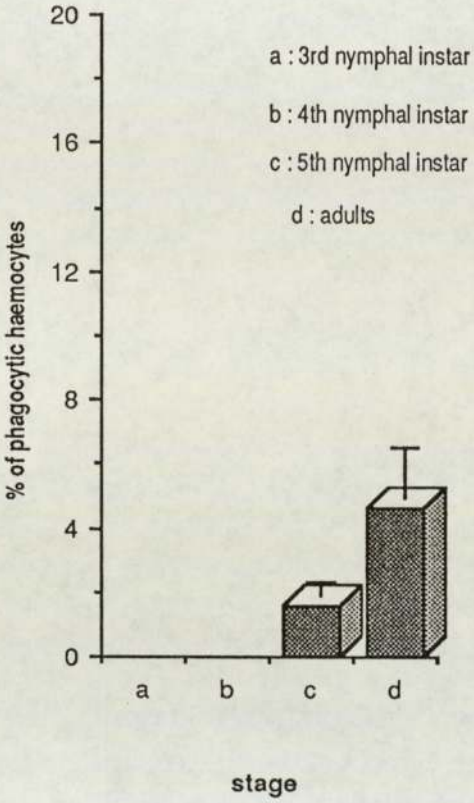
Finally, a further explanation for the differences in phagocytosis of the non-biotic and biotic particles used here may be connected with the physical and chemical properties of the particles themselves. Thus, charcoal and Indian ink particles are much smaller than sheep erythrocytes and this in itself may have some bearing on the rate at which cells can phagocytose these particles. Lackie (1986) has recently drawn attention to the importance of physicochemical characteristics of foreign material such as electrostatic charge and wettability in the processes of haemocyte adhesion and phagocytosis. The surface properties of sheep erythrocytes may more readily permit cell-cell adhesion and phagocytosis, than the inert surfaces of Indian ink particles and charcoal.

The experiments reported here have also examined the ontogeny of the phagocytic immune response. This is the first such systematic study and the results indicate that the phagocytic response of both locust species varies in relation to developmental stage. The phagocytic response of haemocytes to non-biotic particles was very poorly developed or absent altogether in third and fourth nymphal instars, and only the haemocytes of fifth instar and adult locusts displayed consistent levels of particle ingestion. The lack of responsiveness to foreign particles in immature stages may be a consequence of functional immaturity of their haemocytes and further studies are required to determine whether, for instance, the phenoloxydase activity in haemocytes of younger stages is less than that observed in adults cells. An alternative explanation for the poor phagocytic response in immature locusts may be that the amount of particulate material injected was too low to promote a readily observable response. Injection of younger (3rd and 4th instar) stages was problematical however, and high mortality was experienced when "adult" doses of charcoal and particularly Indian ink were administered. The dose employed therefore, was the highest not to cause death. The phagocytic response to SRBC was also significantly less in immature stages than in adult locusts, but haemocytes of all nymphal instars were capable of ingesting xenogeneic erythrocytes much better than inert particles. The lower phagocytic responses of immature individuals may have been due to lower levels of circulating agglutinins following SRBC injection (see chapter three). The precise nature of the interactions between cellular and humoral components of the insect immune system awaits further study.

In addition to the role of the haemocytes in removing injected foreign particles, the pericardial cells and reticular cells of haemopoietic tissue were also shown to be important in particle trapping. Views are divided as to the relative importance of haemopoietic tissue and circulating haemocytes in particle removal. Zachary et al. (1981) maintained that the reticulo-haemopoietic tissue is the corner stone of the antibacterial defence system in L. migratoria. They observed pathogens inside phagocytic tissue but not in circulating haemocytes. In contrast, the studies of Brehelin and Hoffmann (1980) and Cuenot (1986) revealed that phagocytic activity was present in both haemopoietic tissue and circulating haemocytes. A number of authors have also described phagocytic activity in circulating blood cells of insects (Nappi, 1984; Nappi and Carton, 1986; Rizki and Rizki, 1980).

Phagocytic activity in adult and fifth instar haemopoietic tissue results in hypertrophy probably as a result of cellular proliferation (see also autoradiographical studies in chapter two). Zachary et al. (1981) stated that the hypertrophy in haemopoietic tissue resulted primarily from a marked increase in the number of actively dividing undifferentiated cells. Histological studies carried out on phagocytic organs of third and fourth instar nymphs, failed to reveal evidence of charcoal or Indian ink trapping. Further studies employing a wider range of materials are planned in order to assess the importance of haemopoietic tissue in particle trapping in younger stages of locusts.

1-Day Post-injection



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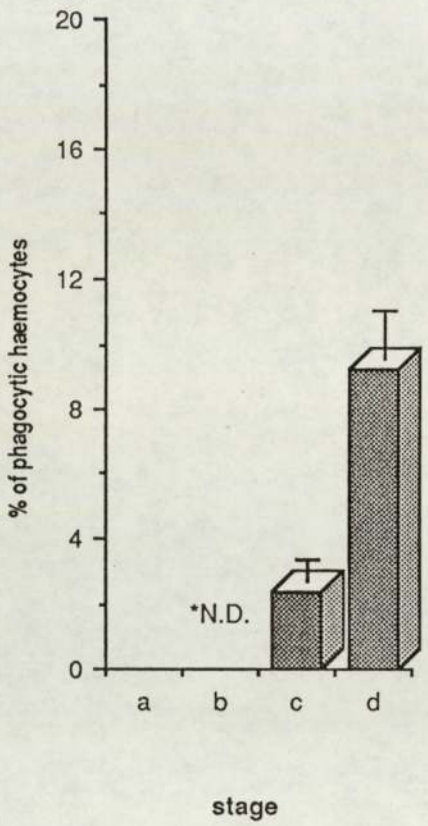

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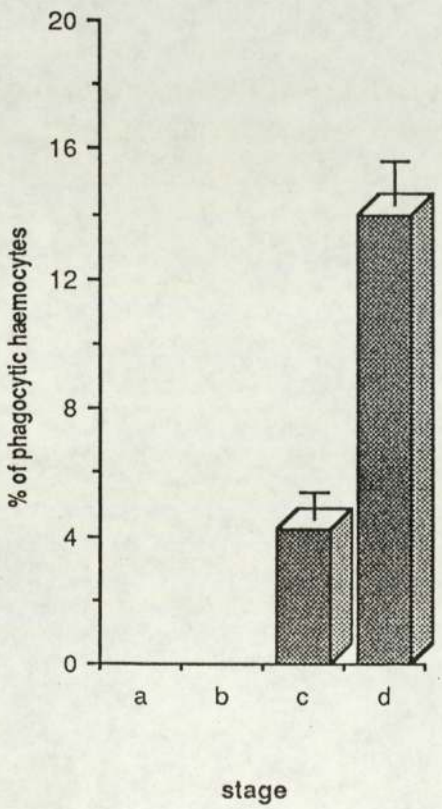

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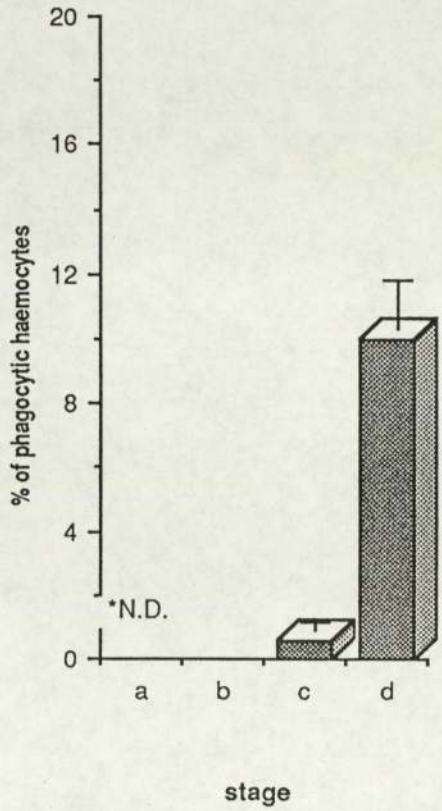

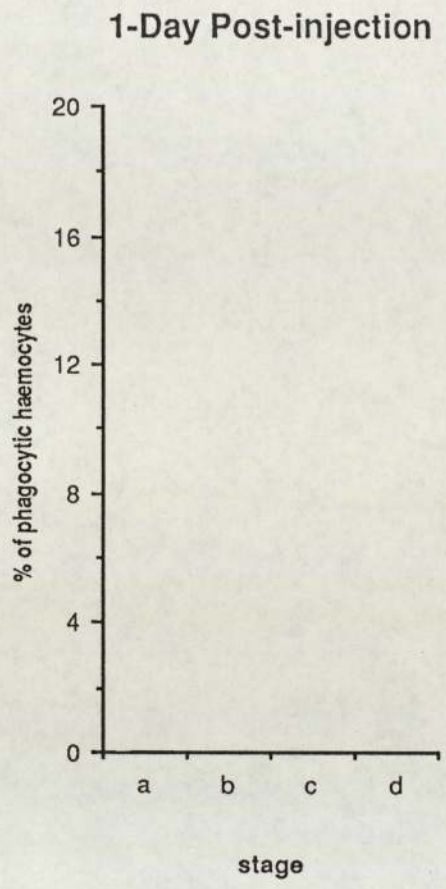
1-Day Post-injection
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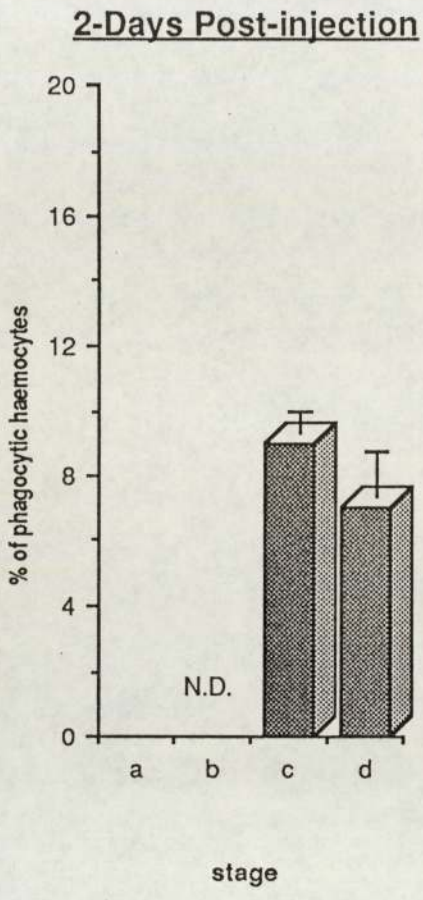
2-Days Post-injection
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% of phagocytic haemocytes
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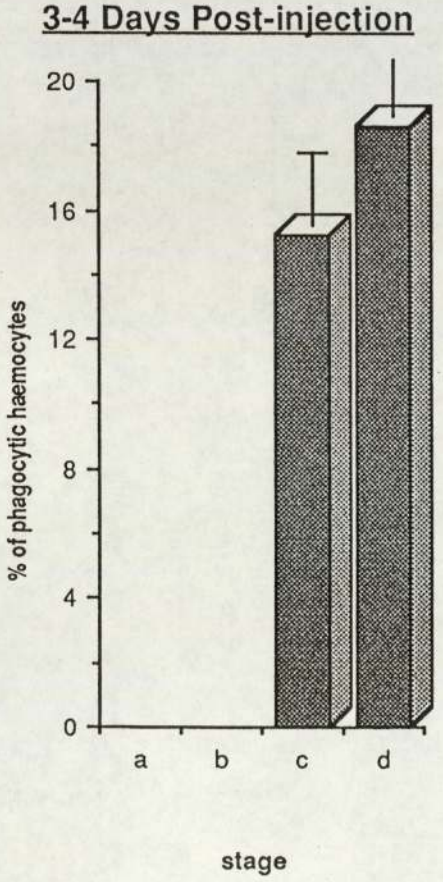
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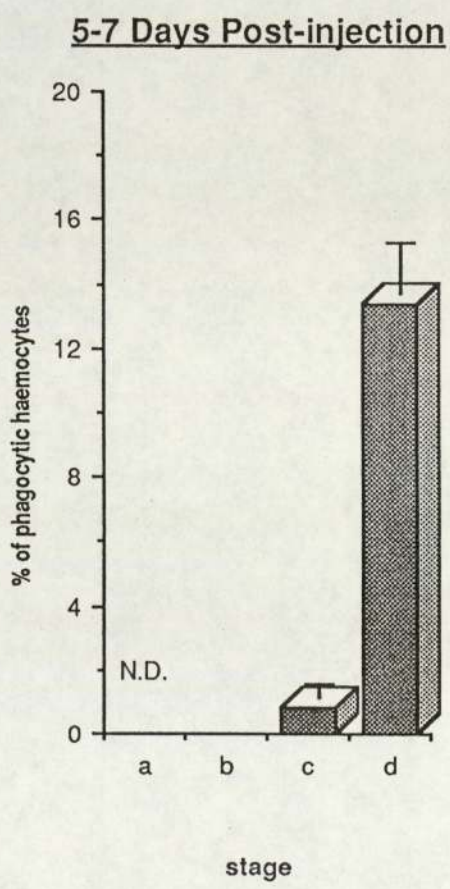
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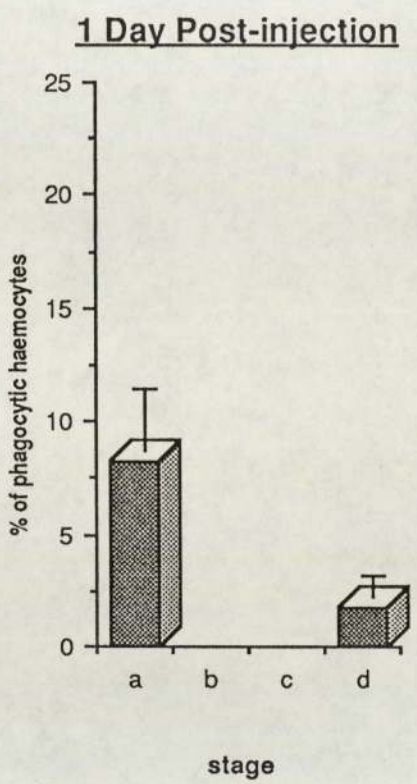
1 Day Post-injection
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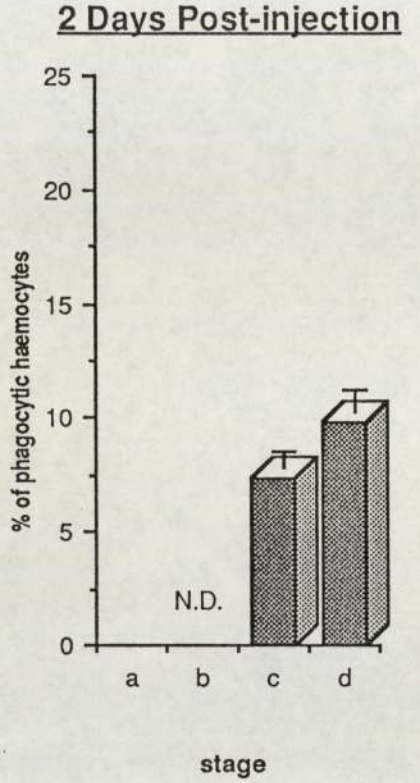
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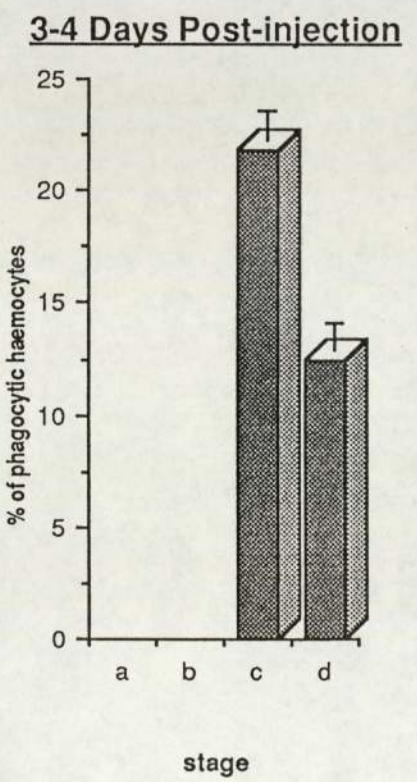
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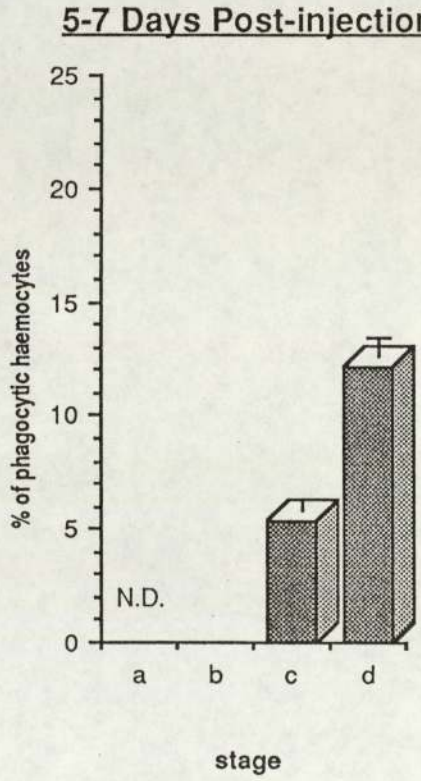
5-7 Days Post-injection
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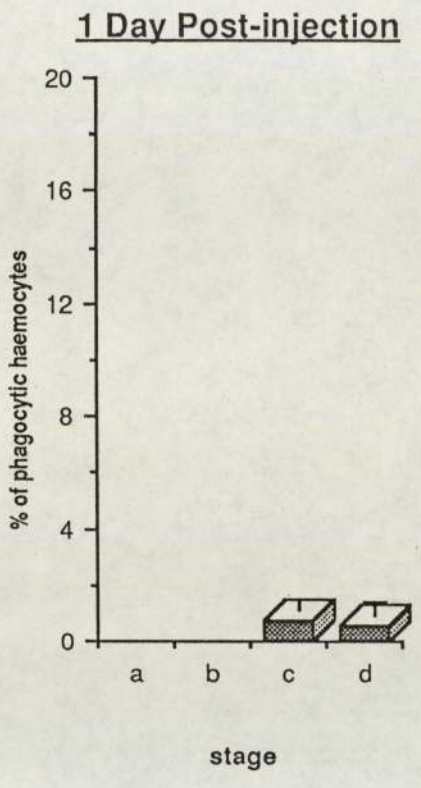
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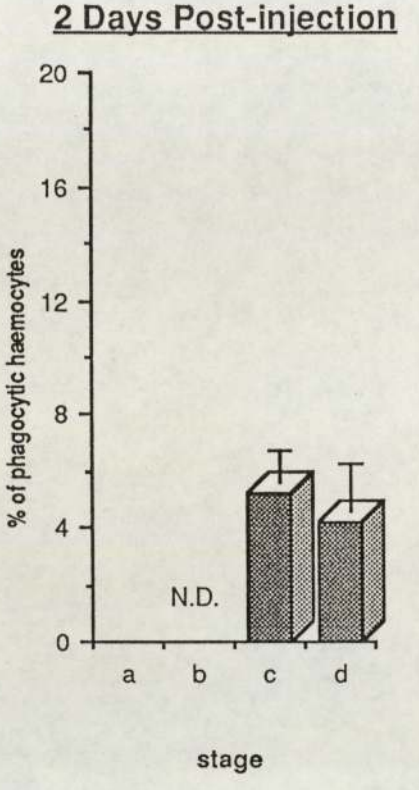
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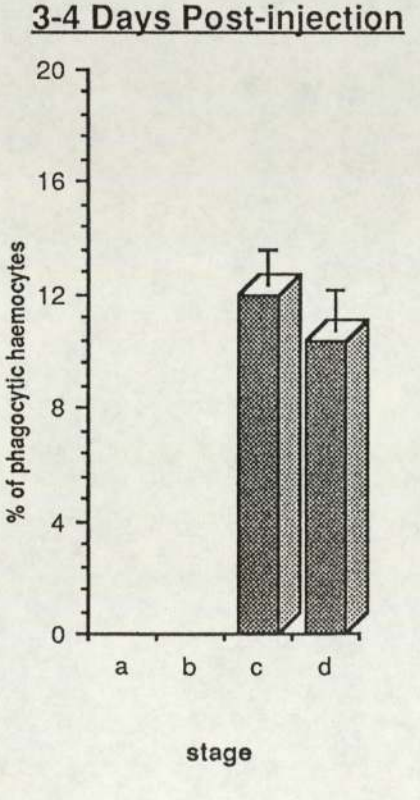
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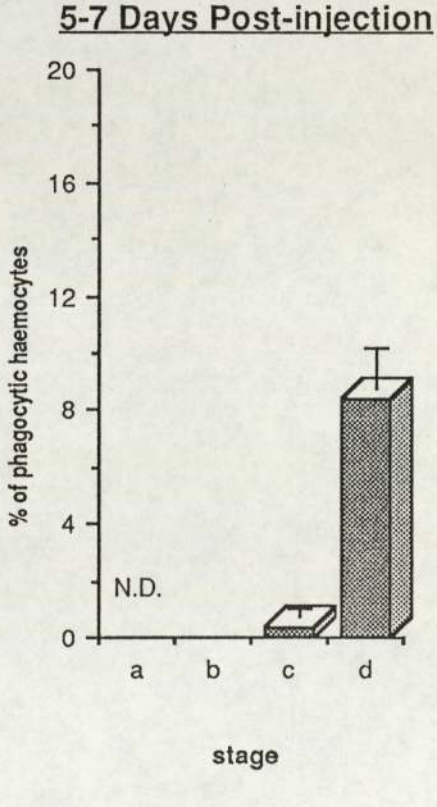
5-7 Days Post-injection
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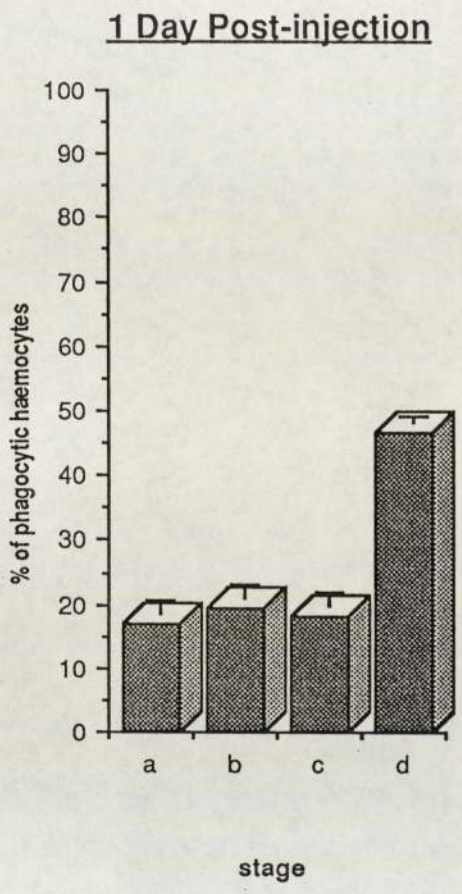
1 Day Post-injection
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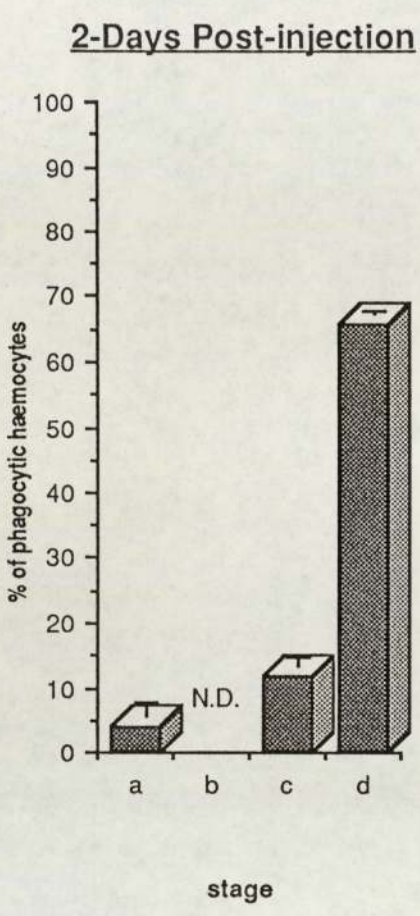
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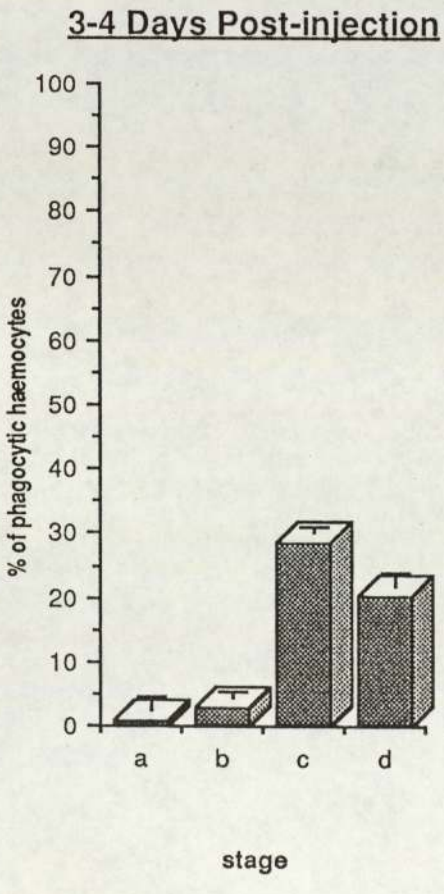
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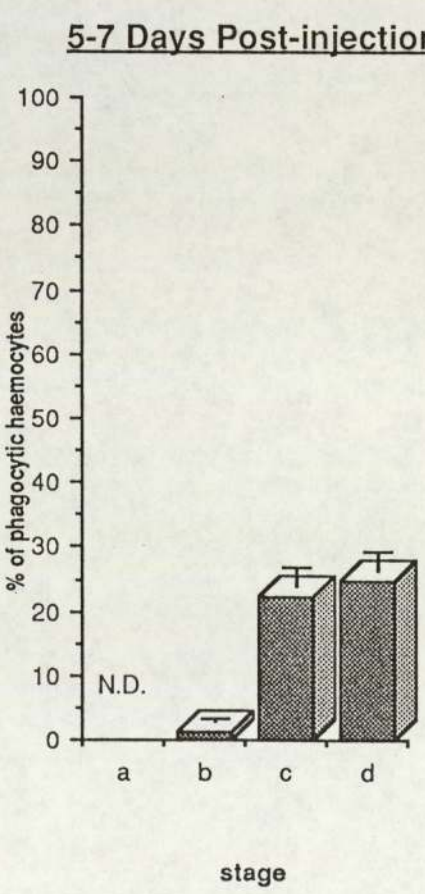
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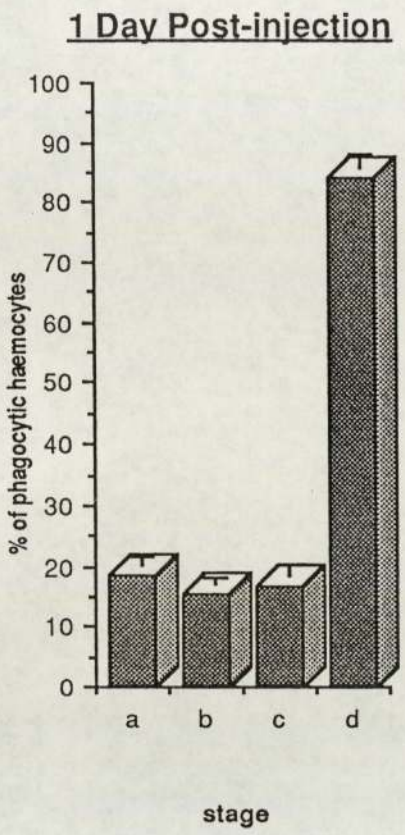
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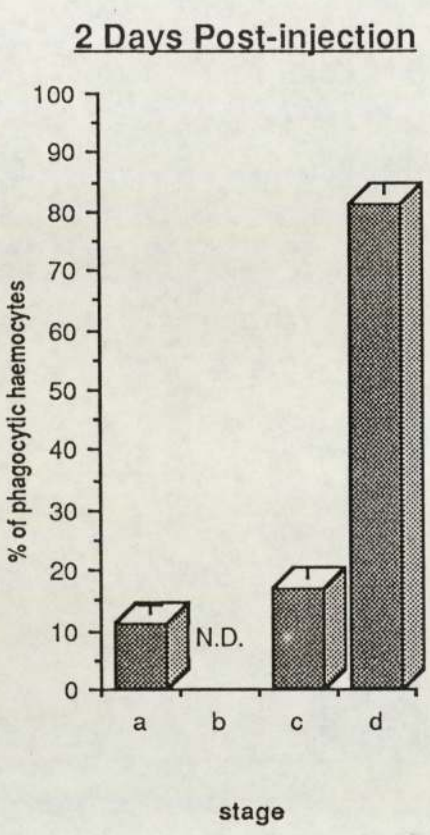
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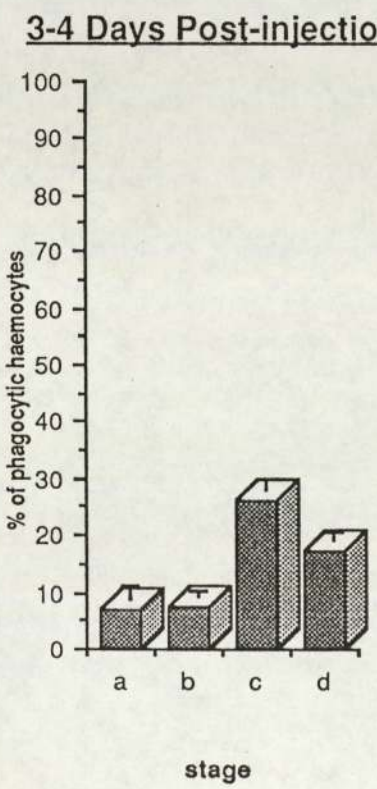
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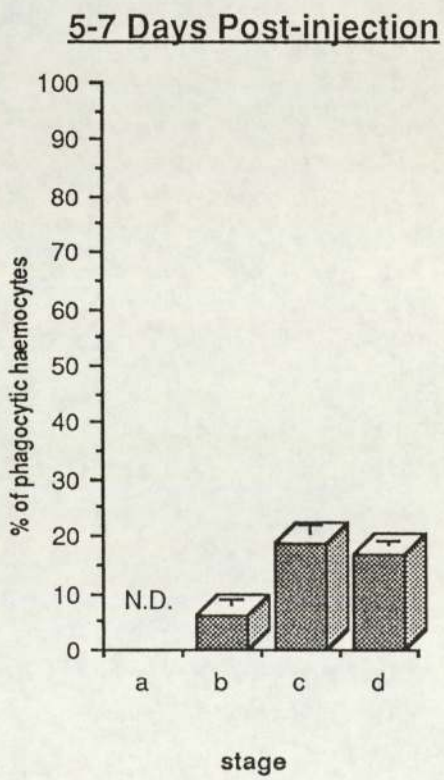
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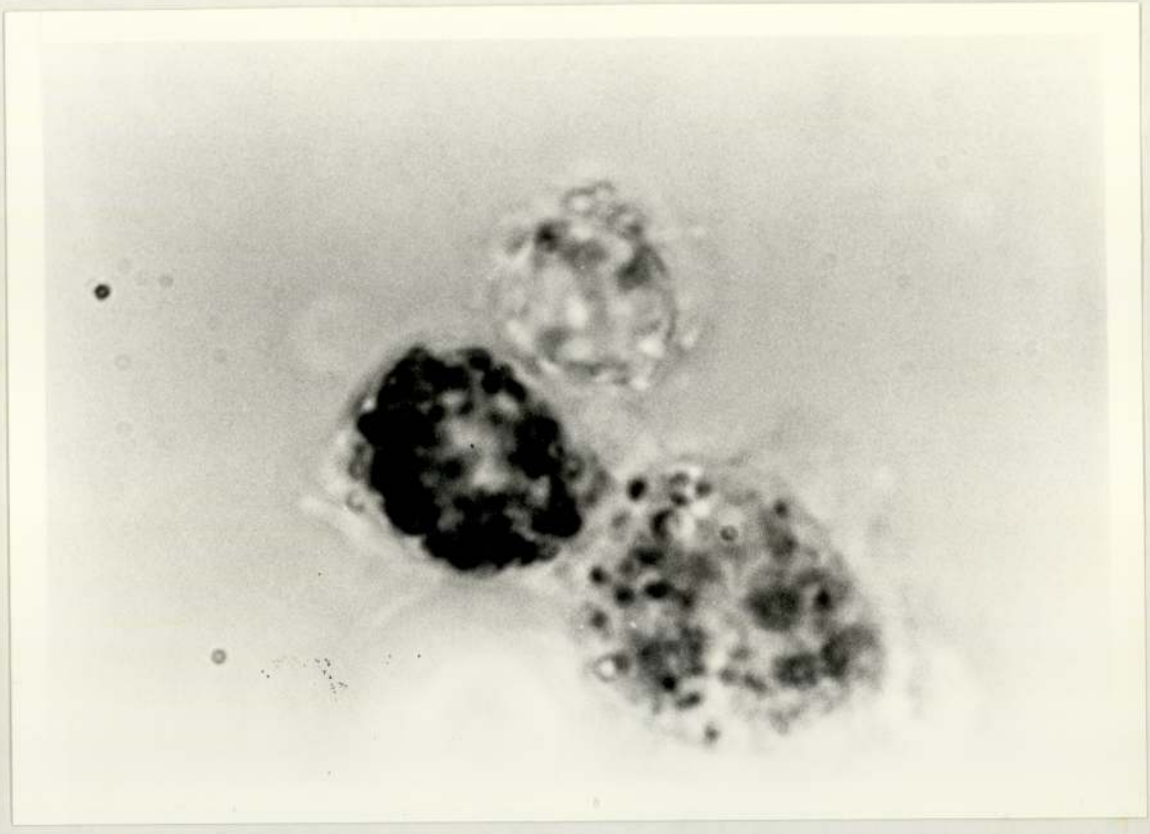

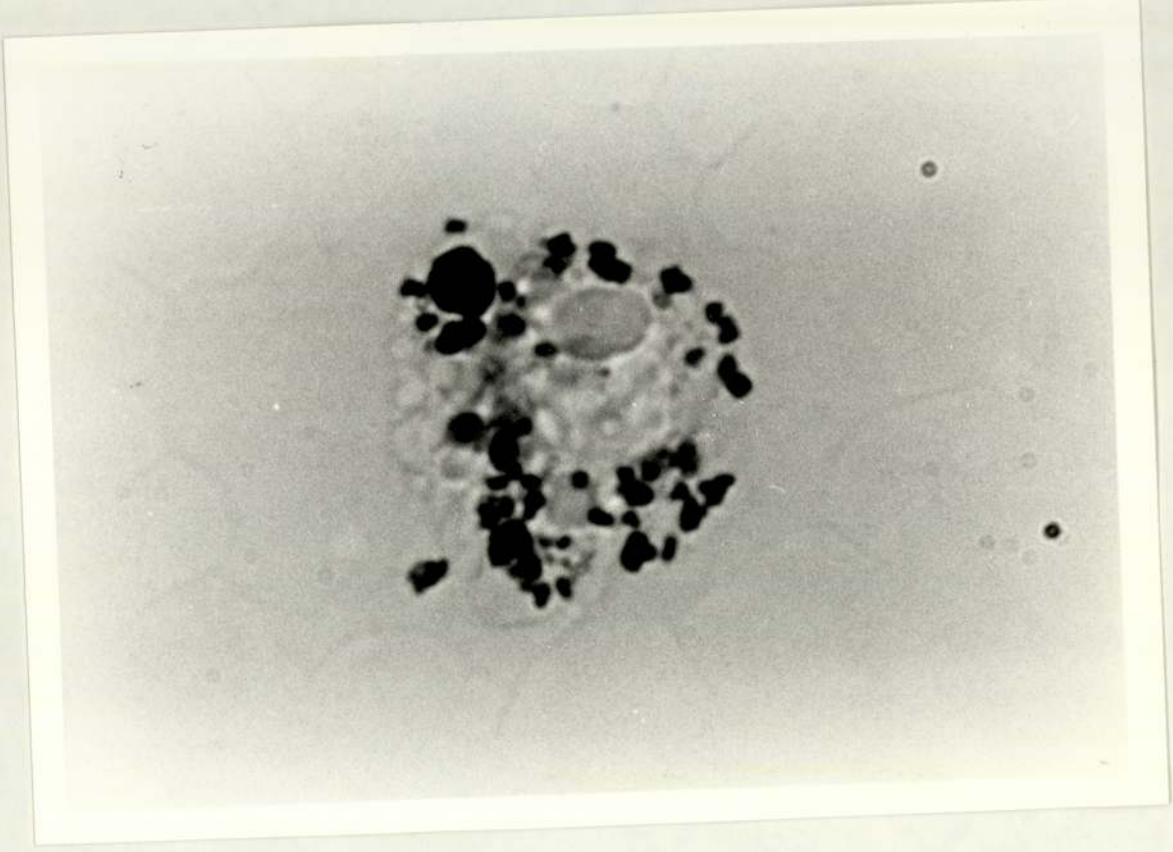

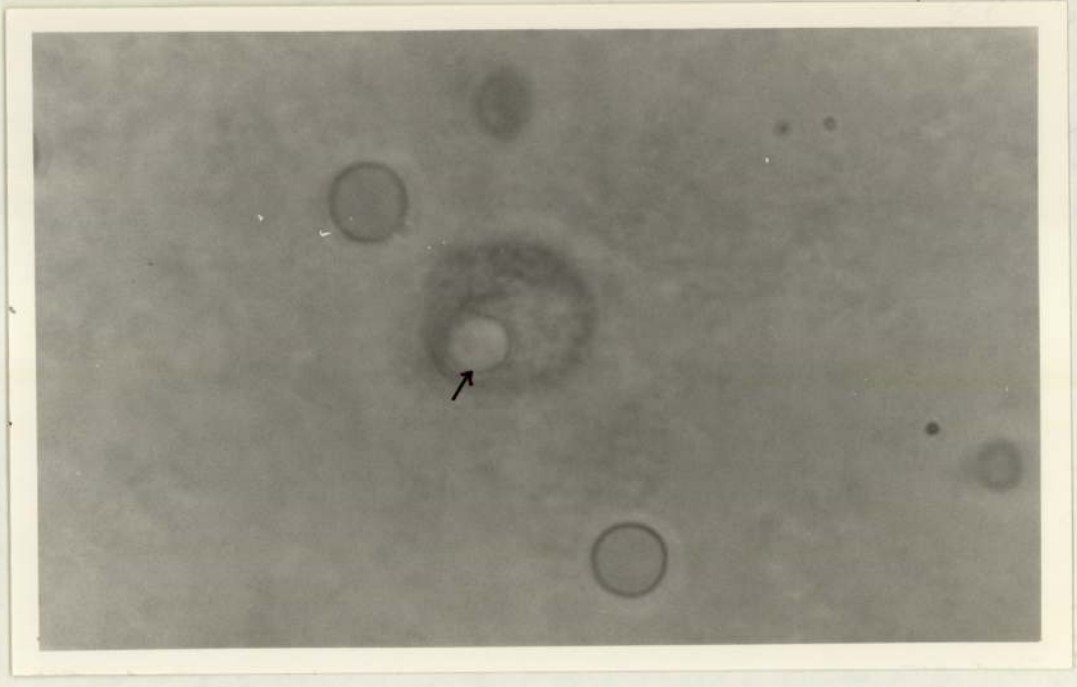

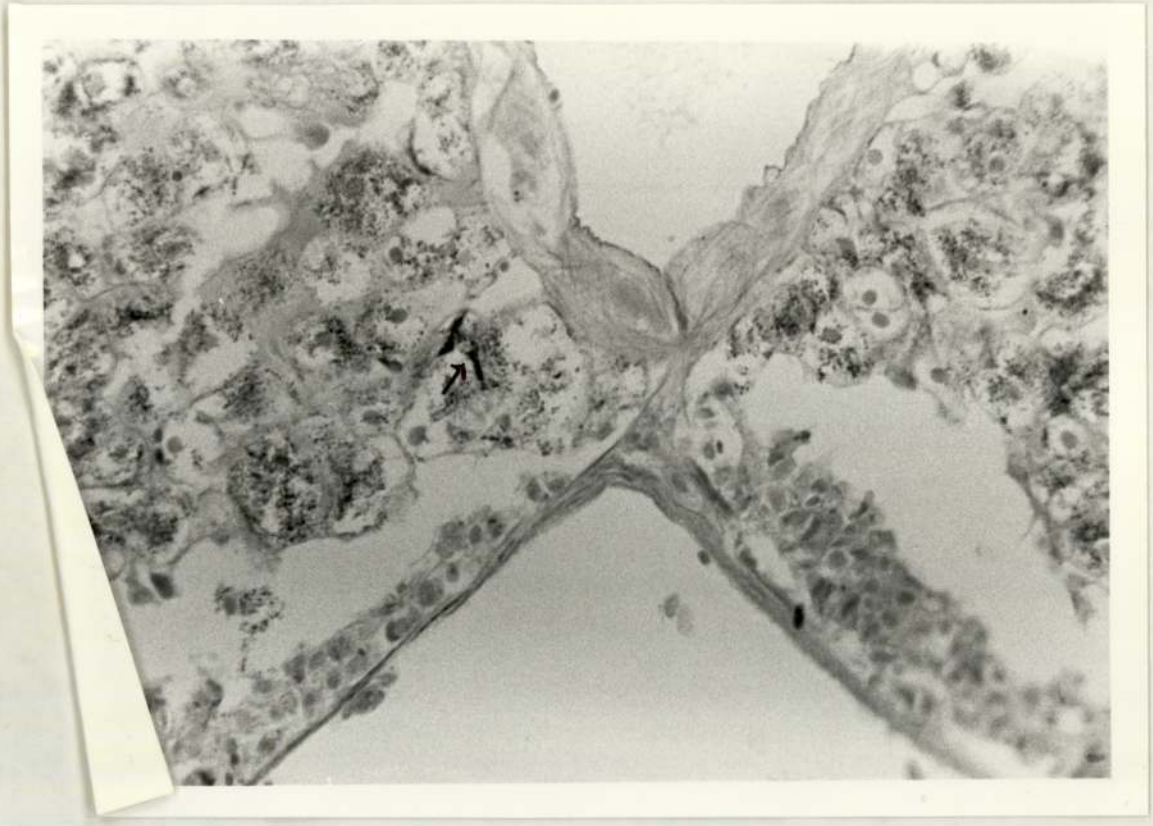

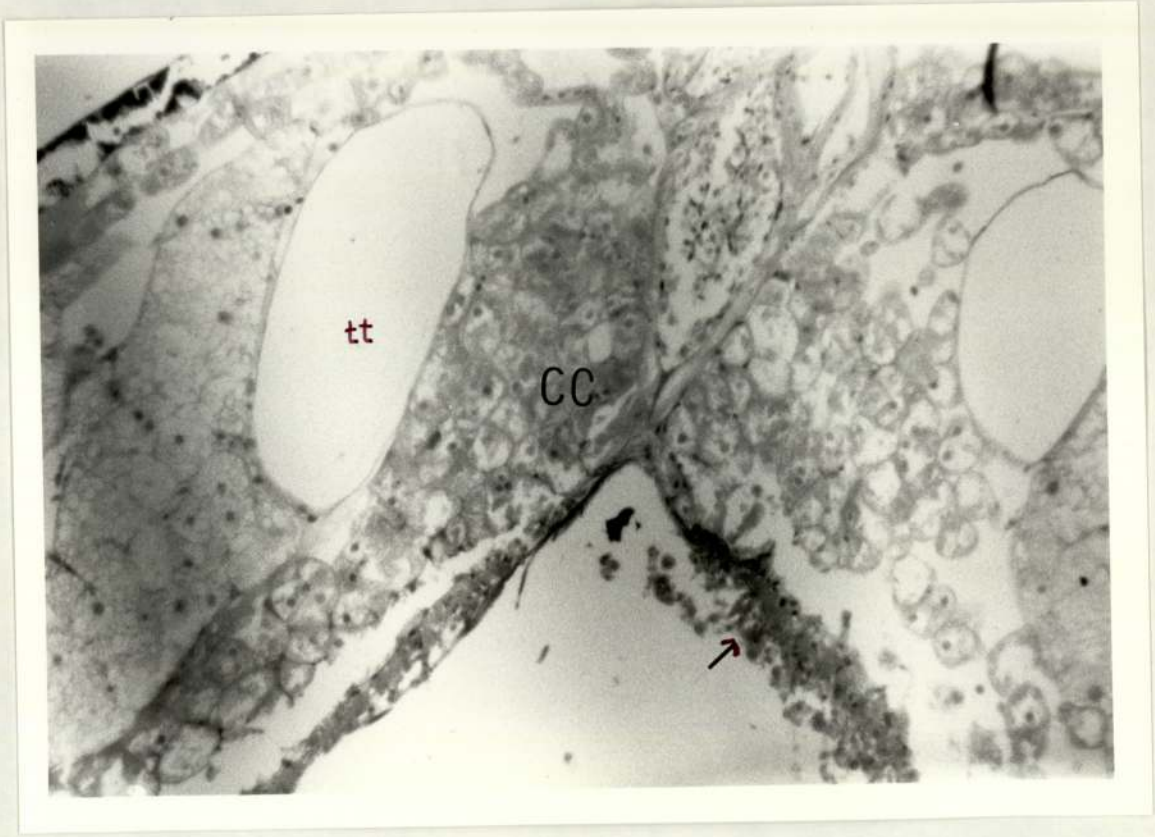
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CHAPTER V

CHEMOTAXIS AND CHEMOKINESIS OF HAEMOCYTES

5.1. Introduction

The first requisite for any cell-mediated defence reaction is contact between the foreign body and the haemocyte surface (Ratcliffe and Rowley, 1979a). Adhesion between the haemocyte and the foreign substratum is the primary event in capsule formation, and those characteristics of the substratum that influence haemocytic adhesion also affect the ability of the insect to mount an effective encapsulation response (Takle and Lackie, 1985). Curtis (1967) proposed that the surface charge of both the substratum and the haemocytes had an important influence on adhesion. Lackie (1983b) later reported that both charge and wettability of substrata are physicochemical parameters that regulate haemocyte adhesion. Following this initial cell contact, the further accumulation of effector and phagocytic cells at sites of antigenic challenge may then depend upon a process called chemotaxis, in which the cells involved undergo oriented movement along a chemical gradient. Chemotactic factors may be of extrinsic (eg. bacterial) or host origin. Cherhas (1973) speculated that a 'chemotactic factor' was present in all insect tissues including haemocytes, and suggested that chemotactic responses in insects are involved in the co-ordination and control of such phenomena as wound healing, phagocytosis, nodule and capsule formation. He proposed that extensive haemocyte autolysis during the first phase of capsule formation would tend to release clouds of this factor into the haemolymph around the developing capsule.

Capsules are formed in insects against a wide range of non-biological and biological objects (Grimstone et al, 1967; Brehelin et al, 1975; Lackie, 1976; Lackie et al, 1985; Salt, 1965; Schmit and Ratcliffe, 1977 and 1978; Robinson and Strickland, 1969; Salt, 1970). The work presented in this chapter addresses the question of whether or not the directional motility of locust haemocytes can be influenced by external factors (antigens) or factors produced by haemocytes during capsule formation.

5.2. Materials and Methods:

Third, fourth and fifth instar nymphs and adults of the migratory locust L. migratoria and the desert locust S. gregaria were used in this study. They were anaesthetised with ether before implantation.

5.2.1. Implantation:

Fragments of sterilized cat gut (about 0.5 cm length) were implanted between the third and fourth sternites of insects that had been surface sterilized with absolute alcohol. Cat gut was used in an attempt to provoke an encapsulation reaction by the locust haemocytes because this material is too large to be phagocytosed. 5 groups of 10 locusts were implanted for each stage of both species. Unfortunately, third and fourth instar locusts died after implantation, so the studies referred to here were carried out on fifth instars and adults only. Locusts were sacrificed and the implanted cat gut removed at intervals post-implantation. Capsules were used as putative sources of chemotactic or chemokinetic agents in the assays described below.

5.2.2. Haemocytes:

Cells were flushed out of chilled insects with 0.5% saline solution containing 5% EDTA (Ethylenediaminotetracetic acid) to avoid clumping of

haemocytes and coagulation of haemolymph. The number of cells was adjusted to 5×10^5 cells/ml.

5.2.3. Chemoattractants:

Four putative chemoattractants were used:

5.2.4. SRBC and E. Coli bacteria:

They were used with the same concentrations as mentioned in previous chapters, and the cells used to test for chemotactic activity were obtained from 5th nymphal instars and adults of both species of locust.

5.2.5. Cat gut capsules:

Capsules at different stages of formation were used. Capsules were removed from insects 24 hrs, 48 hrs, 4 days and 1 week post implantation. Fifth nymphal instars and adults of L. migratoria only were used for this test, since S. gregaria failed to encapsulate the cat gut implants.

5.2.6. Casein:

A 10% casein solution was used because it has previously been shown to be a potent chemoattractant for vertebrate lymphoid cells (Wilkinson, 1972; Gearing and Rimmer, 1986).

5.2.7. Chemotaxis assays:

Two assay systems were used to study the chemotactic and chemokinetic responses of locust cells in response to the agents described above.

1. The agarose gel assay:

The method described by Nelson, Quie and Simons (1975) and applied by Gearing and Rimmer (1986) was employed in which cells migrate from a well cut into an agarose gel towards wells containing chemotactic stimuli. The assay was performed in sterile 60 x 15 mm petri dishes.

Agarose gel was prepared in 100 ml volumes, by dissolving 0.8 g of agarose (BDH) in 38 ml of 0.5% saline solution in a boiling water bath for 20 minutes. 1 ml of 1% foetal caif serum (FCS) (Flow) and 1 ml of combined penicillin and streptomycin (Flow) was added to the gel as it cooled. The FCS was sterilized by filtering through a 0.22 mm disposable millex filter unit. The mixture was delivered to the petri dishes in 10 ml aliquots using a pre-warmed sterile pipette. The gels were then allowed to set on a level table at room temperature and then stored at 4°C until used.

For each assay, two triplicate rows of wells 2.5 mm in diameter and 2.5 mm apart were made in the gel, as shown below, and three petri dishes were used for each test, ie. six replicates for each.

The wells were cut using a pasteur pipette and the gel plugs removed using a hypodermic needle. Fluid which seeped into the wells, from the gel, was removed prior to the assay.

The centre wells, b, received 10 μl of haemolymph which contained 5 x 10^5 cells, and wells, a, were filled with one of the following chemoattractants: 1% SRBC, E. coli bacteria, 10% Casein solution or one

piece of cat gut capsule of a known age immersed in 0.5% saline solution. Wells, c, were used as control. They received 0.5% saline solution in all cases except where cat gut capsules were placed in well a. They received sterile cat gut (1 cm length) in 0.5% saline solution. The plates were covered, and incubated for 18 hours at 25°C and 95% humidity.

Following incubation, the plates were flooded with 5 ml of methanol for 30 minutes, followed by 5 ml of 10% formalin for 30 minutes. This procedure served to fix the cells, and also cleared the gel enabling observation of cells migrating between the gel and the plate surface. Plates were coded and read independently by two separate observers. The scores allocated were 'High' for a strong differential migration towards the chemotactic stimulus; 'Moderate', for differential migration towards the stimulus; and 'None' for no differential migration. It was not possible to quantify the extent of cell migration accurately since irregularities in the cut gel, the dark field lighting conditions, and the differential focusing required to visualise the cells, made precise measurements of the distances travelled by cells impossible.

2. The Micropore Filter Assay:

In an attempt to more precisely quantify the chemotactic response, a modified Boyden chamber assay was used (Boyden, 1962) in which cells migrate from one compartment, through a microspore filter, towards a compartment containing chemoattractants. In the method employed here (after Neuman and Sorg, 1980; Gearing and Rimmer, 1983), the two compartments were formed by the wells of two microtitration plates placed on top of each other, with cellulose acetate filters partitioning the wells.

Assay chambers were prepared by smearing petroleum jelly around the perimeter of both upper and lower test wells. Six wells were used as replicates for each assay amd the same number were used as control. The lower test wells were filled with 1% SRBC solution, E. coli suspension, 10% casein solution in a 0.5% saline or one piece of a known age capsule in 0.5% saline and the lower control wells were filled with either 0.5% saline or 1 mm piece of sterile cat gut in 0.5% saline solution in case of capsules. 5 m filters (Millipore) were then placed over all the lower wells. The upper plate was inverted and placed over the lower plate. Both plates were then clamped tightly together.

30 l of haemolymph solution which contained 5 x 10^5 cells/ml were introduced into each chamber through a hole made in the base of each well with a red-hot hypodermic needle. This number giving an adequate cell density on the filter surface. The whole plate was then covered with aluminium foil to reduce evaporation of liquid, and then placed in a humid incubator at 25°C for 18 hours.

Following incubation, the fluid was removed from the upper wells using a syringe and needle, and replaced with 0.3 ml of methanol, to begin fixation of the cells. After 20 minutes the methanol was removed and the two plates separated. The filters were then fixed in formalin for 20 minutes and stained in Delafields Haematoxylin for five minutes. Filters were then washed with distilled water and each one was mounted in D.P.X. onto microscope slide with the under-surface uppermost.

The number of cells emerging at the lower surface of the 5 m filter was determined microscopically from the mean count of cells in two randomly chosen high-power fields of view. Two counts were made on each of five filters (ie. 10 readings for each experiment). A cell was

counted if, whilst focusing on the filter matrix, any part of that cell was clearly visible. This procedure includes any cells just emerging from the filter pores and those that have completely emerged.

The mean numbers of cells per high-power field of the replicates were compared for stimulus and control filters using Student's 't' test. The chemokinetic effect obtained was expressed by a chemokinetic index calculated as:

$$\text{Chemokinetic index} = \frac{\text{mean number of cells per h.p field with chemokinetic stimulus}}{\text{mean number of cells per h.p field with control stimulus}}$$

5.3. RESULTS

Capsule Formation

Twenty four hours after implantation of cat gut in both 5th instar nymphs and adult Locusta migratoria, the implant was surrounded by loosely aggregated haemocytes. By focusing up and down on the capsule it was just possible to discern several cell layers (Fig.5.1 and 5.2). Evidence of melanization was seen at 48 hr post implantation and, in the present study, was maximal by 1-week. There was no apparent increase in the amount of melanin deposited after this time.

In complete contrast, implants in S. gregaria remained unencapsulated even after two weeks of observation.

5.3.1. The Agarose Gel Assay:

I. L. Migratoria

SRBC had a chemotactic effect on haemocytes of both fifth instar nymphs and adults. The response was slightly more marked in fifth

instar nymphs than in adults. In nymphs, 4 out of 6 experiments gave medium chemotaxis scores and 2 gave high scores, whereas in adults the scores were 2 high and 3 medium, one trial gave a negative score (Table 5.1).

E. coli bacteria also had a marked chemotactic effect on both adults and fifth instar haemocytes. In adults 3 out of the 6 experiments gave a high chemotaxis score, whilst 2 experiments gave medium scores. Four of the six trials carried out using fifth instar haemocytes gave medium scores (Table 5).

10% casein solution resulted in a weak chemotactic effect with both 5th nymphal instars and adult haemocytes. For both stages, four out of six trials gave negative results and the remaining two gave only modern chemotactic responses.

Table 5.2 shows that cat gut capsules removed at 48-hrs and 4-days post-implantation produced a positive chemotactic effect in both 5th instar and adult haemocytes. The 48-hr capsules exerted a stronger attractive effect than did 4-day capsules. In fifth instar nymphs, 48-hr capsules gave 3 medium and 2 high chemotaxis scores and the 4-day capsules gave 4 medium chemotaxis score. 24-hr and 1-week old capsules had much less effect, 24-hr capsules gave 1 medium score and the same score was obtained with 1-week old capsules. In adults 48-hr capsules gave 3 medium and 2 high scores, 4 day capsules gave 3 medium and 1 high score but 24-hr capsules were completely negative and 1-week capsules gave 2 medium chemotaxis score. Six trials were carried out in each case.

II. S. gregaria

Haemocytes from S. gregaria showed no differential migration towards any of the chemoattractants used: SRBC, E. coli bacteria and Casein failed to produce positive chemotaxis scores in both 5th nymphal instars and adults (Table 5). Since no capsules were formed in response to cat gut implants in S. gregaria, it was not possible to examine the putative chemoattractant effect of capsule cells and their products.

5.3.2. The Micropore Filter Assay:

The results of this assay are presented in Tables 5.3 and 5.4.

I. L. migratoria

As in the gel assay, SRBC produced a significant cellular response with both 5th nymphal instars and adult haemocytes. With adult cells, a highly significant chemokinetic index of 2.8 was obtained and 5th instar cells gave a significant chemokinetic index of 1.9 (Table 5.3).

E. coli bacteria also generated significant chemokinetic activity. Fifth instar and adult cells gave chemokinetic indices of 2.0 and 2.3 respectively (Table 5.3).

In the case of Casein, no chemokinetic activity was generated with either 5th instar or adult haemocytes and the chemokinetic indices were both non-significant (Table 5.3).

Experiments in which cat gut capsules of different ages were used in the lower chamber of assay chambers showed that both 48-hr old and 4-day old capsules produced chemokinetic activity in both 5th instar and adult haemocytes. Adult cells were more responsive with chemokinetic

indices of 3.9 for 48-hr old capsules, and 3.3 for 4-day old capsules. In fifth instars the chemokinetic indices for 48-hr capsules and 4-day old capsules were 2.3 and 2.1 respectively. The chemokinetic indices generated by 24-hr old capsules and 1-week old capsules of both 5th instars and adults were non-significant (Table 5.4).

II. S. gregaria

SRBC produced a significant chemokinetic effect in both 5th instar and adult haemocytes. The chemokinetic index for adult cells (2.7) was slightly higher than that seen for fifth instar cells (2.1). In contrast, E. coli and Casein did not generate any chemokinetic activity in either 5th instar or adult cells (Table 5.3).

5.4. DISCUSSION

In vertebrates, immune responses such as delayed type hypersensitivity reactions, allograft rejection and reactions to parasitic infections are often accompanied by a massive localised influx of cells. The accumulation of effector cells and phagocytes at sites of antigenic challenge is dependent upon a process termed chemotaxis, in which cells undergo oriented movement along a chemical gradient. The factors which stimulate the migration, homing and accumulation of leucocytes at inflammatory sites may be of extrinsic (eg. bacterial) or host origin (reviewed in Gallin and Quie, 1978). The latter group includes factors such as immunoglobulin and complement components, together with soluble mediators known as lymphokines. Lymphocyte derived chemotactic factors have been described in mammals and birds and, more recently, in amphibians and fish (Gearing and Rimmer, 1985; St G Howell, 1987).

In contrast, chemotaxis in insects has not been studied in detail but is thought to be involved in encapsulation responses, wound healing and phagocytosis (Harvey and Williams, 1961; Ratcliffe, 1986; Gotz, 1986, and Lackie, 1986). The work presented in the present chapter has adapted methods originally used for vertebrate cells for use with insect haemocytes. The use of these techniques has allowed us to study a range of agents capable of stimulating chemotactic or chemokinetic responses.

The agarose method for *in vitro* studies on chemotaxis was found to have several advantages (see also Cutler, 1974, and Notes, 1976). The technique allows direct observation of migrating cells and the direction of migration can be easily and rapidly assessed. Whilst some problems were experienced during attempted fixation and staining of migrating cells, further development of the technique should permit the identification of the predominant cell types associated with phagocytosis, encapsulation and wound healing reactions. In the present study, haemocytes of *L. migratoria* were shown to exhibit a positive chemotactic response towards SRBC and *E. coli*.

Chemotaxis of insect haemocytes to foreign objects was reported by Vey et al. (1975) who employed microcinematographic techniques to observe unidirectional locomotion of insect blood cells towards fungal spores. Detection of and migration towards foreign micro-organisms or cells may be along a concentration gradient of molecules released by the foreign material itself or by haemocytes which have already contacted and reacted to the foreign agent by producing soluble chemotactic factors. The precise nature of factors chemotactic for insect haemocytes is unknown, but Renwrantz (1986) has suggested that, as with mammalian leucocytes (Wilkinson, 1981), invertebrate phagocytes may

undergo chemotaxis along peptide gradients. Polypeptides and more complex proteins form part of the membrane structure of cells and may be released in sufficient quantities to attract haemocytes towards foreign invaders. An alternative chemoattractant source may be provided by surface carbohydrates which form part of membrane polysaccharides, glycoproteins or glycolipids. These proposals require more detailed study.

In contrast to vertebrate leucocytes (Wilkinson and McKay, 1972, and Gearing and Rimmer, 1985), haemocytes from both species of locust failed to respond strongly to casein. Casein is a protein molecule with a large number of hydrophobic groupings. In mammalian cells the surface hydrophobicity of casein is thought to be important in contributing to its chemotactic properties. The addition of hydrophobic groupings to the chemotactically inert bovine serum albumin causes it to become progressively more chemoattractive. It may be that in the case of locust haemocytes, the mechanism of action of chemotactic factors is not the same as that seen in vertebrate cells.

The modified Boyden chamber assay is widely referred to as a chemotaxis assay. However, some critics have pointed out that since the only direction of movement examined is that towards the "chemoattractant" in the lower chamber, a false impression of oriented movement may be obtained, and that cells may simply be moving more in all directions. In the present study, therefore, the effects observed in the modified Boyden chamber assay are referred to as chemokinesis, although this does not necessarily preclude chemotaxis.

The experiments reported here demonstrate that chemokinetic activity of L. migratoria haemocytes could be detected in the presence

of SRBC and E. coli but not in the presence of casein. Chemokinetic activity was also observed when haemocytes of L. migratoria were exposed to encapsulated cat gut implants. Maximal activity was observed when cells were incubated with capsules which were removed from the hosts at 2-days and 4-days post-implantation. One day and seven day old capsules produced less chemokinetic activity. The reasons for the differences observed may be related to the timed release of chemotactic factors by encapsulating cells. In the case of encapsulating reactions it has been widely proposed that the first haemocytes to make contact with the implant release a chemotactic factor (or factors) which is attractive to other haemocytes. This factor would diffuse outwards through the haemolymph so that a concentration gradient is established with the capsule in the region of highest concentration (presumably the relatively sluggish blood circulation of insects would be conducive to the establishment of localised chemotactic gradients). After sufficient quantities of haemocytes have accumulated at the site of the implant, the process of melanization begins and no further haemocytes recruitment is observed (Cherbas, 1973; Ratcliffe and Gagen, 1976; Schmit and Ratcliffe, 1976; Satz et al., 1976; Gagen, 1976; Hillen, 1977, and Gotz, 1986).

Whilst the encapsulation reaction was strong in L. migratoria, cat gut implants in S. gregaria failed to evoke a response even after two weeks of observation. As with other aspects of the immune system studied here, the reaction of S. gregaria to foreign material appeared to be less highly developed than that of L. migratoria. The reasons for this apparent difference are not clear, since morphologically at least, the haemocyte types are the same in both species. It is perhaps surprising, therefore, that these cells do not appear to function with equal efficiency in the two types of locust.

Whilst chemotactic and chemokinetic responses have both been described here, it is not yet clear what role these activities play in encapsulation and how the haemocytes of insects communicate with each other. The cellular response in many insect species seems to involve the interaction of granulocytes and plasmatocytes in a biphasic process (Ratcliffe and Rowley, 1979, and Gotz, 1986). In many species studied, the first cells to make contact (presumably at random) with the foreign implant are the granulocytes which then undergo degranulation. The discharged material sticks to the foreign surfaces and to blood cells and the attached granulocytes disintegrate. This process appears to attract plasmatocytes which adhere to the foreign body, flatten out and establish cell to cell contact involving desmosomal attachments. Melanization follows and appears to originate at the surface of the foreign object (Gotz, 1976). The process of melanization involves the prophenoloxidase activating system - a complex cascade of enzymes which is ultimately responsible for the initiation of melanin synthesis (Soderhall and Smith, 1986). The possible role of the prophenoloxidase system in phagocytosis has already been referred to in Chapter Four, and it is not inconceivable that it may also be involved in the chemotactic behaviour of haemocytes during capsule formation. Further work is necessary to examine the chemotactic properties of extrinsic and intrinsic factors on insect haemocytes.

Species	Stage	Chemotactic Stimulus	Chemotaxis Scores		
			None	Medium	High
L. migratoria	5th nymphal instars	SRBC	0	IIII	II
L. migratoria	Adults	SRBC	I	III	II
L. migratoria	5th nymphal instars	E. coli	II	IIII	0
L. migratoria	Adults	E. coli	I	II	III
L. migratoria	5th nymphal instars	Casein	IIII	II	0
L. migratoria	Adults	Casein	IIII	II	0
S. gregaria	5th nymphal instars	SRBC	IIII	II	0
S. gregaria	Adults	SRBC	IIII	II	0
S. gregaria	5th nymphal instars	E. coli	IIII	II	0
S. gregaria	Adults	E. coli	IIIII	I	0
S. gregaria	5th nymphal instars	Casein	IIIII	I	0
S. gregaria	Adults	Casein	IIIII	I	0

Table 5.1 The chemotactic movement of locust haemocytes in response to SRBC, E. coli bacteria and casein in the agarose gel assay.

Species	Stage	Chemotactic stimulus	Chemotaxis scores		
			None	Medium	High
L. migratoria	5th nymphs	24hr cat gut cape	IIIII	I	0
		48hr cat gut cape	I	III	II
		4-days	II	IIII	0
		1-week	IIIII	I	0
L. migratoria	Adults	24hr cat gut cape	IIIIII	0	0
		48hr cat gut cape	I	III	II
		4-days	II	III	I
		1-week	IIII	II	0

Table 5.2 The chemotactic movement of L. migratoria haemocytes in response to cat gut capsules in the agarose gel assay.

Chemokinetic Stimulus	Stage and Species	Mean no. of cells per H.P. field ± S.E. (control)	Mean no. of cells per H.P. field ± S.E. (treated)	Chemokinetic index	Significance of difference
SRBC	5th nymphal instar *L. migratoria*	53.2 ± 5.09	105.3 ± 2.32	1.9	0.01
	Adult *L. migratoira*	32.6 ± 2.91	93.8 ± 4.56	2.8	0.01
E. coli	5th nymphal instar *L. migratoria*	48.1 ± 1.93	96.3 ± 6.78	2.0	0.01
	Adult *L. migratoria*	45.9 ± 2.90	106.2 ± 4.82	2.4	0.01
Casein	5th nymphal instar *L. migratoria*	45.7 ± 5.06	57.2 ± 4.51	1.2	N S
	Adult *L. migratoria*	46.1 ± 8.22	48.6 ± 7.90	1.05	N S
SRBC	5th nymphal instar *S. gregaria*	46.1 ± 1.16	98.7 ± 2.44	2.1	0.05
	Adult *S. gregaria*	32.5 ± 2.33	88.3 ± 3.67	2.7	0.01
E. coli	5th nymphal instar *S. gregaria*	58.1 ± 3.07	63.1 ± 2.81	1.08	N S
	Adult *S. gregaria*	61.7 ± 2.59	67.3 ± 7.46	1.09	N S
Casein	5th nymphal instar *S. gregaria*	44.8 ± 7.22	53.4 ± 3.01	1.1	N S
	Adult *S. gregaria*	65.2 ± 2.67	64.3 ± 4.82	0.9	N S

Table 5.3 The chemokinetic movement of locust haemocytes in response to SRBC, *E. coli* bacteria and Casein in the modified Boyden chamber filter assay.

Age of capsule	Stage and Species	Mean no. of cells per H.P. field ± S.E. (control)	Mean no. of cells per H.P. field ± S.E. (treated)	Chemokinetic Index	Significance of difference
24 hrs.	5th nymphal instar *L. migratoria*	33 ± 6.90	44.6 ± 5.82	1.3	N S
48 hrs.	5th nymphal instar *L. migratoria*	49.6 ± 1.08	116.9 ± 2.31	2.3	0.01
4-days	5th nymphal instar *L. migratoria*	49.9 ± 2.27	106.3 ± 4.71	2.1	0.05
1-week	5th nymphal instar *L. migratoria*	50.5 ± 5.69	70.8 ± 6.28	1.4	N S
24 hrs.	Adult *L. migratoria*	27.8 ± 4.88	28.1 ± 6.06	1.01	N S
48 hrs	Adult *L. migratoria*	30.7 ± 1.12	122.7 ± 0.09	3.9	0.001
4-days	Adult *L. migratoria*	30.5 ± 0.23	103.2 ± 1.65	3.3	0.001
1-week	Adult *L. migratoria*	34.2 ± 9.89	69.2 ± 7.88	2.02	N S

Table 5.4 The chemokinetic movement of the haemocytes of 5th numphal instars and adults of *L. migratoria* in response to cat gut capsules of different ages in the modified Boyden chamber filter assay.

CHAPTER SIX

GENERAL DISCUSSION

In contrast to the vertebrates, few studies have been carried out on the ontogeny of immunity in invertebrates. The work presented in this thesis has attempted to examine the emergence of immunocompetence in two species of locust.

The present study has revealed that the different haemocyte types appear early in ontogeny at the third and fourth nymphal instar stages, and that the relative percentages of cells present remains constant throughout development. Studies on insect blood cells are made difficult by a number of factors including the problems of clotting, extraction of adequately sized samples from small animals and changes in morphology (e.g. degranulation) which can sometimes occur *in vitro*. Staining characteristics can also make identification of cells difficult (Ratchliffe et. al., 1986). The present study made use of phase contrast microscopy in order to distinguish different cell types, but even this method can pose problems especially if cells have phagocytosed foreign particles which may obscure cytoplasmic detail. Future studies on the identification and functional properties of insect haemocytes could be greatly improved by the use of recently developed cell separation techniques (e.g. using continous Percoll gradients as described by Ratcliffe et. al. 1986) and by the use of (monoclonal) antibodies or lectin binding specificities to identify surface determinants unique to different haemocyte types. These approaches have already been successfully employed in addelids (Roch and Valembois, 1978),

molluscs (Schoenberg and Cheng, 1980; Yoshino and Granath, 1983, 1985; Renwrantz et al., 1985) and tunicates (Schlumpberger et al., 1984). In insects, Rizki and Rizki (1983) and Nappi and Silvers (1984) have utilised lectin-binding (wheat germ agglutinin) to study haemocytes in Drosophila. Changes in the haemocyte profile were also noted in the present study following antigenic challenge of both immature and adult locusts. Some preliminary studies carried out here suggested that antigenic challenge was followed by proliferative changes in the haemopoietic tissues. Pathak (1986) has recently drawn attention to the lack of information concerning the factors influencing the blood picture of insects. It would be of interest of discover what effect, if any, removal or functional impairment (e.g. by irradiation) of the haemopoietic tissue had on immune responsiveness. The endocrine control of haemocyte numbers could also be investigated, since agents which disturb the normal functioning of the insect defence system might be of use in experimental manipulation of the immune response and in insect pest control.

Agglutinins to foreign erythrocytes and bacteria were studied in chapter three. However, there is still much to be learned about the origin of these factors and their functional role in vivo. The present study demonstrated that haemolymph with a high haemagglutinin titre was able to promote the in vitro phagocytosis of erythrocytes. However, further studies are required both to separate and identify the active haemolymph fraction(s) responsible for agglutination and to determine their cellular origin. Flourescent antibodies raised against insect haemagglutinins and

bacterial agglutinins could be used to identify those cells or tissues showing intracytoplasmic or membrane bound agglutinins.

In a recent review Ratcliffe (1986) has emphasised the need for a clearer understanding of the interactions which exist between cellular and humoral components of insect immunity. These interactions are likely to be important in a range of immunological events including phagocytosis and encapsulation which were examined in chapters four and five. A number of soluble factors have been implicated in these processes including extrinsic agents (such as lipopolysaccharide and β 1-3 glucan) and factors produced by the host cells themselves (e.g. agglutinins, components of the prophenoloxidase system and factors involved in wound healing). The chemotaxis and chemokinetic assays applied here may prove useful in investigating the influence of these and other factors on haemocyte behaviour.

One of the major aims of this thesis was, wherever possible, to study the developmental appearance of various immunological reactions. In some cases this was made difficult or impossible by the susceptibility of younger stages to experimental intervention. As outlined in the introduction, an ontogenetic approach to insect immunity may prove useful in understanding fundamental aspects of non-self recognition and discovering how animals with small number of immune cells and a less sophisticated capacity for immunological recognition are able to cope with a hostile environment. Such studies may also be of use in devising biological control programmes. In vertebrates there is considerable evidence that premature exposure to antigens can result in immunological

tolerance. Whether a similar phenomenon exists in insects is not known, but exposure to pathogenic strains of bacteria or fungi during early developmental stages may kill immature insects outright or induce a state of tolerance which would render them more susceptible to pathogenic attack as adults. The potential for the use of pathogens as biological control agents probably exceeds that of many other pest management tactics (Evans, 1984). However, the development of pathogen-based systems of pest management has been impeded by problems of identification, culture and registration of microbial agents for use in control programs. In addition, there is still much to be learned concerning the factors governing insect host responses to pathogens. An important area for future studies on locust immunity ought to be concerned with their reactions to a range of bacterial, viral and protozoan pathogens and to microparasites such as nematode worms. Such studies should provide a clearer understanding of insect immunity and increase prospects for the successful biological control of locusts and other insect pests.

Stage	Time post injection	Granulocytes % ± S.E.	Coagulocytes % ± S.E.	Plasmatocytes % ± S.E.
3rd instar nymphs	Control	35 ± 1.832	51.4 ± 2.122	13.6 ± 1.200
	1-day	47.3 ± 0.465***	31.5 ± 0.631***	21.2 ± 0.544**
	2-days	52.1 ± 0.231***	35.7 ± 0.718***	12.2 ± 0.871
	4-days	41.9 ± 0.425*	47.8 ± 1.654	10.3 ± 0.201*
4th instar nymphs	Control	26.6 ± 2.098	48.6 ± 2.714	24.8 ± 2.046
	1-day	55.2 ± 2.339***	14.4 ± 0.866***	30.4 ± 3.024
	3-days	63.4 ± 2.768***	33 ± 2.931*	3.6 ± 1.023***
	5-days	61.7 ± 2.887***	35.1 ± 2.834*	3.2 ± 1.216***
5th instar nymphs	Control	31 ± 0.989	50.2 ± 0.491	18.8 ± 0.308
	1-day	40 ± 0.828**	45.3 ± 0.529**	14.7 ± 0.402**
	2-days	38.7 ± 1.242	47.2 ± 3.024	14.1 ± 0.242
	4-days	40 ± 0.828**	46.1 ± 0.494**	13.9 ± 0.282**
	7-days	48.5 ± 0.244***	35.4 ± 0.636***	16.1 ± 0.441**
Adult	Control	43.6 ± 0.447	45.8 ± 0.316	10.6 ± 0.316
	1-day	45.1 ± 0.367*	40.7 ± 0.342**	14.2 ± 0.463**
	2-days	44.8 ± 0.242	41.6 ± 0.722	13.6 ± 0.396
	4-days	44.2 ± 0.406	43 ± 0.648*	12.8 ± 0.412*
	7-days	50.1 ± 0.441***	41.6 ± 0.406**	8.3 ± 0.244**

Table 2.1 Percentages of the three types of haemocytes in different developmental stages of L. migratoria at different times post-injection of 1% SRBC.

* Significant.
** Highly significant.
*** Very highly significant.

Stage	Time post injection	Granulocytes % ± S.E.	Coagulocytes % ± S.E.	Plasmatocytes % ± S.E.
3rd instar nymphs	Control	41 ± 0.964	56.5 ± 2.106	2.5 ± 0.734
	1-day	60.8 ± 1.384***	23.2 ± 1.520***	16 ± 0.628***
	2-days	56.8 ± 2.782**	16.75 ± 4.01***	28.8 ± 1.614***
	4-days	50.2 ± 1.023**	22.7 ± 2.316***	27.1 ± 1.030***
4th instar nymph	Control	40.5 ± 1.241	54 ± 1.311	5.5 ± 1.571
	1-day	48.6 ± 1.115**	31.8 ± 2.954***	19.6 ± 2.786*
	3-days	63.4 ± 1.848***	22 ± 0.942***	14.6 ± 1.680*
	5-days	61.1 ± 1.516***	20.9 ± 1.637***	18 ± 2.137**
5th instar nymph	Control	38.2 ± 0.316	52.4 ± 0.556	9.4 ± 0.374
	1-day	38.8 ± 0.273	50 ± 0.561*	11.2 ± 0.316*
	2-days	39.2 ± 0.719	48.9 ± 0.913	11.9 ± 0.496
	4-days	39.7 ± 0.616	45 ± 0.254***	15.3 ± 0.543**
	7-days	38 ± 0.667	46.7 ± 0.524**	15.3 ± 0.254***
Adult	Control	36.4 ± 0.512	49.8 ± 0.552	13.8 ± 0.923
	1-day	40.3 ± 0.435**	48 ± 0.418*	11.7 ± 0.122
	2 days	41.4 ± 0.638	47.6 ± 1.428	11.0 ± 0.837
	4-days	42.1 ± 0.514	46.7 ± 0.514*	11.2 ± 0.158*
	7-days	44 ± 0.612 **	48.1 ± 0.441*	7.9 ± 0.223**

Table 2.2 Percentages of the three types of haemocytes in different developmental stages of S. gregaria at different times post-injection of 1% SRBC.

Stage	Time post injection	Granulocytes % ± S.E.	Coagulocytes % ± S.E.	Plasmatocytes % ± S.E.
3rd instar nymphs	Control	35 ± 1.832	51.4 ± 2.122	13.6 ± 1.200
	1-day	45.6 ± 3.120*	30.8 ± 2.835***	23.6 ± 1.033***
	2-days	56.6 ± 3.040*	24 ± 1.692***	19.4 ± 1.620*
	4-days	48.3 ± 3.926**	33.6 ± 2.096***	18.1 ± 2.617
4th instar nymphs	Control	26.6 ± 2.098	48.6 ± 2.714	24.8 ± 2.046
	1-day	30.6 ± 3.120	43 ± 2.591	26.4 ± 2.661
	3-days	50.8 ± 1.972***	30.8 ± 3.815*	18.4 ± 2.821
	5-days	46.7 ± 3.864***	42.6 ± 3.284	10.7 ± 2.304***
5th instar nymphs	Control	31 ± 0.989	50.2 ± 0.491	18.8 ± 0.308
	1-day	44.8 ± 0.412***	46.8 ± 0.412**	8.4 ± 0.636***
	2-days	45.1 ± 0.618	45.7 ± 0.913	9.2 ± 1.239
	4-days	45.7 ± 0.863***	47.6 ± 0.519*	6.7 ± 0.863***
	7-days	55.1 ± 0.565***	33.7 ± 0.474***	11.2 ± 0.339***
Adult	Control	43.6 ± 0.447	45.8 ± 0.316	10.6 ± 0.316
	1-day	51.2 ± 0.254**	40.7 ± 0.616**	8.1 ± 0.494*
	2-days	51.4 ± 0.827	41 ± 1.631	7.6 ± 1.458
	4-days	50.7 ± 0.863**	41.1 ± 0.212**	8.2 ± 0.316**
	7-days	60.6 ± 0.583***	27.3 ± 0.331***	12.1 ± 0.681

Table 2.3 Percentages of the three types of haemocytes in different developmental stages of L. migratoria at different times post-injection of E. coli.

Stage	Time post injection	Granulocytes % ± S.E.	Coagulocytes % ± S.E.	Plasmatocytes % ± S.E.
3rd instar nymphs	Control	41 ± 0.532	56.5 ± 0.167	2.5 ± 0.327
	1-day	62.2 ± 0.441***	28.7 ± 0.214***	9.1 ± 0.167***
	2-days	61.7 ± 0.634***	30.7 ± 0.880***	7.6 ± 0.349***
	4-days	53.2 ± 0.801***	43.4 ± 0.604***	3.4 ± 0.551
4th instar nymphs	Control	40.5 ± 0.416	54 ± 0.567	5.5 ± 0.871
	1-day	55.7 ± 0.842***	42.1 ± 0.408***	2.2 ± 0.506*
	3-days	58.1 ± 0.645***	45.7 ± 0.815**	0.2 ± 0.333**
	5-days	52.3 ± 0.339***	45.8 ± 0.764**	1.9 ± 0.790*
5th instar nymphs	Control	38.2 ± 0.316	52.4 ± 0.556	9.4 ± 0.374
	1-day	39.1 ± 0.494	50 ± 0.254*	10.9 ± 0.282*
	2-days	39.5 ± 0.634	49.6 ± 0.863	10.9 ± 0.138
	4-days	40 ± 0.815	48.2 ± 0.316**	11.8 ± 0.291**
	1-week	49.3 ± 0.331***	35.6 ± 0.583***	15.1 ± 0.681**
Adult nymphs	Control	36.4 ± 0.512	49.8 ± 0.552	13.8 ± 0.923
	1-day	40.3 ± 0.412**	45.6 ± 0.406**	14.1 ± 0.331
	2-days	42.4 ± 0.528	45.9 ± 0.983	11.7 ± 1.213
	4-days	43.7 ± 0.863**	46 ± 0.141**	10.3 ± 0.223*
	1-week	50.1 ± 0.681***	38.7 ± 0.474***	11.2 ± 0.339*

Table 2.4 Percentages of the three types of haemocytes in different developmental stages of S. gregaria at different times post injection of E. coli.

Stage	Time Post-injection	Mean agglutination titre ± S.E.	
		L. migratoria	*S. gregaria*
3rd nymphal instar	Control	0	0
	1-day	1.2 ± 0.200	0
	2-days	1.4 ± 0.244	0
	4-days	1.4 ± 0.244	0
4th nymphal instar	Control	0	0
	1-day	1.4 ± 0.244	0.4 ± 0.244
	3-days	1.6 ± 0.509	0.6 ± 0.244
	5-days	1.6 ± 0.509	0.4 ± 0.244
5th nymphal instar	Control	0	0.6 ± 0.244
	1-day	1.8 ± 0.199	0.6 ± 0.244
	2-days	2.6* ± 0.623	0.5 ± 0.186
	4-days	4.0 ± 0.316	0.4 ± 0.244
	7-days	4.8* ± 0.199	0.6 ± 0.244
Adult	Control	0.6 ± 0.244	0.4 ± 0.244
	1-day	2.4* ± 0.244	0.6 ± 0.244
	2-days	2.8 ± 0.136	0.7 ± 1.232
	4-days	3.4** ± 0.244	0.6 ± 0.244
	7-days	3.8** ± 0.199	0.4 ± 0.244

Table 3.1 Agglutination of SRBC by the haemolymph of different developmental stages of *L. migratoria* and *S. gregaria* at different times post-antigenic challenge.

In this and subsequent tables control = mean background level of agglutination for all saline-injected locusts.

Stage	Time post-injection	Mean agglutination titre ± S.E.	
		L. migratoria	*S. gregaria*
3rd nymphal instar	Control	1.8 ± 0.374	0.8 ± 0.374
	1-day	2.0 ± 0.547	1.4 ± 0.244
	2-days	4.8* ± 0.244	2.0 ± 0
	4-days	2.8* ± 0.374	1.0 ± 0.316
4th nymphal instar	Control	2.1 ± 0.374	0.4 ± 0.244
	1-day	2.6 ± 0.200	1.6 ± 0.244
	3-days	4.0* ± 0.244	1.6 ± 0.244
	5-days	2.0 ± 0.316	0.8 ± 0.200
5th nymphal instar	Control	0.2 ± 0.200	0
	1-day	0.4 ± 0.244	0
	2-days	2.6 ± 0.602	0
	4-days	4.8* ± 0.734	0
	7-days	6.0** ± 0.894	0
Adult	Control	0	0
	1-day	0	0
	2-days	0	0
	4-days	1.0 ± 0.316	0
	7-days	1.2 ± 0.374	0

Table 3.2 Agglutination of MRBC by the haemolymph of different developmental stages of *L. migratoria* and *S. gregaria* at different times post-antigenic challenge.

Stage	Time post-injection	Mean agglutination titre ± S.E.	
		L. migratoria	S. gregaria
3rd nymphal instar	Control	0	0
	1-day	0	0
	2-days	0	0
	4-days	0	0
4th nymphal instar	Control	0	0
	1-day	0	0
	3-days	0	0
	5-days	0	0
5th nymphal instar	Control	1.8 ± 0.200	1.8 ± 0.200
	1-day	2.0 ± 0	2.8 ± 0.200
	2-days	1.6 ± 0.242	2.0 ± 0.737
	4-days	1.4 ± 0.400	2.2 ± 0.583
	7-days	0	0
Adult	Control	0	0
	1-day	0	0
	2-days	0	0
	4-days	0	0
	7-days	0	0

Table 3.3 Agglutination of SRBC by the haemolymph of different developmental stages of L. migratoria and S. gregaria at different times post-antigenic challenge.

Stage	Time post-injection	Mean agglutination titre ± S.E.	
		L. migratoria	*S. gregaria*
3rd nymphal instar	Control	0	0
	1-day	0	0
	2-days	0	0
	4-days	0	0
4th nymphal instar	Control	0	0
	1-day	0.8 ± 0.374	0
	3-days	2.2 ± 0.583	0
	5-days	1.8 ± 0.199	0
5th nymphal instar	Control	3.0 ± 0.447	0
	1-day	3.8 ± 0.373	0.2 ± 0.199
	2-days	4.18 ± 0.272	0.2 ± 0.199
	4-days	5.2* ± 0.199	0.2 ± 0.199
	7-days	5.0* ± -	0.2 ± 0.199
Adult	Control	1.0 ± 0.316	0
	1-day	1.8 ± 0.199	0.2 ± 0.199
	2-days	1.8 ± 0.327	0.2 ± 0.242
	4-days	2.4* ± 0.244	0.2 ± 0.199
	7-days	4.0** ± 0.316	0.2 ± 0.199

Table 3.4 Agglutination of *E. coli* by the haemolymph of different developmental stages of *L. migratoria* and *S. gregaria* at different times post-antigenic challenge.

	Percentage of phagocytic locusta Haemocytes containing the following number of Intracellular FSRBC.			
FSRBC Treatment	None	1-3	4-6	7-9
Haemolymph-Incubated Unwashed	48.8 ± 0.362	23.4 ± 0.242	20 ± 1.127	7.8 ± 3.012
Haemolymph-Incubated Washed	27.4 ± 0.617	24.6 ± 0.138	39.8 ± 0.244	8.2 ± 2.063
Saline Incubated Control	78.2 ± 0.138	1.8 ± 0.199	12.3 ± 1.236	7.7 ± 1.837

Table 3.5 Opsonising effect of immune haemolymph *in vitro*

Each figure represents the mean value of 6 replicates cultures ± S.E.

Stage	Time Post injection	Locusta migratoria None % ± S.E.	Locusta migratoria Small amount % ± S.E.	Locusta migratoria Large amount % ± S.E.	Schistocerca gregaria None % ± S.E.	Schistocerca gregaria Small amount % ± S.E.	Schistocerca gregaria Large amount % ± S.E.
3rd nymphal instar	1 - day	100	0	0	100	0	0
	2 - days	100	0	0	100	0	0
	4 - days	100	0	0	100	0	0
4th nymphal instar	1 - day	100	0	0	100	0	0
	3 - days	100	0	0	100	0	0
	5 - days	100	0	0	100	0	0
5th nymphal instar	1 - day	98.4 ± 0.400	1.6 ± 0.400	0	100	0	0
	2 - days	97.6 ± 0.748	2.2 ± 0.583	0.2 ± 0.200	91.0 ± 0.632	7.6 ± 0.244	1.4 ± 0.600
	4 - days	95.8 ± 0.583	1.6 ± 0.509	2.6 ± 0.812	84.8 ± 2.135	12.8 ± 2.289	2.4 ± 0.871
	7 - days	99.4 ± 0.244	0.6 ± 0.244	0	99.2 ± 0.374	0.4 ± 0.244	0.4 ± 0.4
Adults	1 - day	95.4 ± 1.568	4.6 ± 1.568	0	100	0	0
	2 - days	90.8 ± 2.709	5.6 ± 1.503	3.6 ± 1.435	93.0 ± 1.549	5.8 ± 1.428	1.2 ± 0.799
	4 - days	86 ± 1.949	6.8 ± 1.280	7.2 ± 1.240	81.4 ± 0.979	11 ± 1.9493	7.6 ± 1.469
	7 - days	90 ± 1.244	3.4 ± 1.520	6.6 ± 1.166	86.6 ± 1.536	6.6± 0.871	6.8 ± 1.529

Table 4.1 Phagocytosis of charcoal by the haemocytes of the two species of locust.

Stages	Time post injection	Locusta migratoria			Schistocerca gregaria		
		None % S.E.	Small amount % ± S.E.	Large amount % ± S.E.	None % ± S.E.	Small amount % ± S.E.	Large amount % ± S.E.
3rd nymphal instar	1 - day	91.8 ±	6.2 ± 2.738	2 ± 1.987	100	0	0
	2 - days	100	0	0	100	0	0
	4 - days	100	0	0	100	0	0
4th nymphal instar	1 - day	100	0	0	100	0	0
	3 - days	100	0	0	100	0	0
	5 - days	100	0	0	100	0	0
5th nymphal instar	1 - day	100	0	0	99.2 ± 0.374	0.8 ± 0.374	0
	2 - days	92.6 ± 0.678	7.4 ± 0.678	0	94.8 ± 1.200	5.2 ± 1.200	0
	4 - days	78.2 ± 1.529	18.2 ± 1.303	3.6 ± 0.400	88.0 ± 1.378	4.6 ± 1.166	7.4 ± 0.812
	7 - days	94.6 ± 0.509	4.8 ± 0.489	0.6 ± 0.244	99.6 ± 0.244	0.4 ± 0.244	0
Adults	1 - day	98.2 ± 0.860	1.8 ± 0.860	0	99.4 ± 0.4	0.6 ± 0.400	0
	2 - days	90.2 ± 1.562	8.6 ± 0.979	1.2 ± 0.800	95.8 ± 1.685	4.2 ± 1.685	0
	4 - days	87.6 ± 1.435	8.6 ± 0.927	3.8 ± 1.240	89.6 ± 1.630	6.6 ± 1.435	3.8 ± 1.240
	7 - days	87.8 ± 1.019	5.4 ± 0.400	6.8 ± 0.734	91.6 ± 1.691	3.8 ± 0.734	4.6 ± 1.400

Table 4.2 Phagocytosis of indian ink by the haemocytes of the two species of locust.

		Locusta migratoria			Schistocerca gregaria		
Stage	Time post injection	None % ± S.E.	One % ± S.E.	More than one % ± S.E.	None % ± S.E.	One % ± S.E.	More than one % ± S.E.
3rd nymphal instar	1 - day	83.2 ± 1.324	15.1 ± 1.783	1.7 ± 2.301	81.4 ± 2.701	15.6 ± 1.023	3.0 ± 0.544
	2 - days	95.8 ± 0.903	3.4 ± 1.021	0.8 ± 1.792	89.0 ± 1.937	6.2 ± 1.348	4.8 ± 1.106
	4 - days	99.1 ± 2.353	0.9 ± 1.923	0	92.8 ± 1.535	6.4 ± 2.021	0.8 ± 0.912
4th nymphal instar	1 - day	80.4 ± 1.494	14.4 ± 1.709	5.2 ± 0.963	84.6 ± 1.590	8.8 ± 0.795	6.6 ± 1.115
	3 - days	97.0 ± 0.702	2.4 ± 0.674	0.6 ± 0.397	92.4 ± 2.25	4.8 ± 0.854	2.8 ± 0.795
	5 - days	98.6 ± 0.397	1.3 ± 0.244	0.1 ± 0.023	93.7 ± 0.244	5.1 ± 0.353	1.2 ± 0.489
5th nymphal instar	1 - day	81.8 ± 2.083	18.2 ± 2.083	0	83.4 ± 1.886	15.8 ± 1.959	0.8 ± 0.374
	2 - days	88 ± 0.632	9.6 ± 0.927	2.4 ± 0.678	82.0 ± 2.626	14.8 ± 1.772	3.2 ± 1.319
	4 - days	71.6 ± 1.288	22.2 ± 0.860	6.2 ± 1.241	73.8 ± 1.854	18.2 ± 1.907	8.0 ± 1.600
	7 - days	77.8 ± 2.059	12.6 ± 2.908	9.6 ± 1.029	81.4 ± 1.166	12.2 ± 1.685	6.4 ± 1.503
Adult	1 - day	53.2 ± 0.800	34.4 ± 0.871	12.4 ±0.812	15.8 ± 1.378	45.4 ± 1.631	38.8 ±2.353
	2 - days	34.2 ± 1.319	54.2 ± 1.019	11.6 ±0.509	18.8 ± 0.800	39.6 ± 1.435	41.6 ±1.691
	4 - days	79.6 ± 1.720	20.4 ± 1.720	0	82.6 ± 1.503	14.2 ± 1.113	3.2 ± 0.860
	7 - days	75.4 ± 3.265	24.4 ± 3.187	0.2 ± 0.200	83.4 ± 0.509	15.8 ± 0.374	0.8 ± 0.489

Table 4.3 Phagocytosis of SRBC by the haemocytes of the two species of locust.

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