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SUBSTRATE ADHESION AND GAP JUNCTION FORMATION BY INSECT HEMOCYTES *IN VITRO*

by
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Department of Zoology

Submitted in partial fulfillment
of .ne requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
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ABSTRACT

Circulating insect hemocytes become adherent to foreign surfaces and to one another during immune and wound healing responses. Hemocytes form various types of intercellular junctions, including gap junctions, as they encapsulate large foreign objects in the hemocoel. This study focuses on two aspects of hemocyte behaviour *in vitro*: hemocyte/substrate adhesion and gap junction formation.

Cockroach (*Periplaneta americana*) hemocytes resuspended in medium containing purified lipophorin, either in the presence or absence of Ca^{2+}, remained non-adherent to glass coverslips and retained a discoid morphology *in vitro* for at least 30 minutes. In contrast, hemocytes incubated either with or without Ca^{2+} for 30 minutes in lipophorin-deficient plasma, BSA, or saline alone adhered and flattened onto coverslips. The finding that lipophorin stabilized hemocytes *in vitro* is important since to date it has been difficult to maintain hemocytes in a non-adhesive state *in vitro*.

Calf serum inhibited hemocyte adhesion in the presence and absence of Ca^{2+}. Hemocyte adhesion was inhibited by the human plasma lipoprotein apoB-100, but not by apoA-I, apoA-II, apoC-I, apoC-II, apoE, or mouse IgG. Of eight synthetic peptides with sequences corresponding to short regions (15 to 43 amino acids) of human apoB-100, one peptide corresponding to amino acids 4154-4189 inhibited hemocyte adhesion. Human apoB-89, a truncated form of apoB-100 lacking the region

containing 4154-4189, was also active, indicating that one or more additional sites exist on apoB-89 which are involved in inhibiting hemocyte adhesion.

Freeze-fracture replicas of hemocyte aggregates fixed 5 minutes after bleeding were seen to contain E-face particles coalescing to form gap junctional plaques. Dye passage was detected between carboxyfluorescein diacetate-labelled and unlabelled hemocytes within 3 minutes of bleeding, when the cells made contact as they flattened rapidly onto coverslips. Dye-coupling was detected in the absence of Ca^{2+}, indicating that involvement of Ca^{2+}-dependent cell adhesion molecules is not a prerequisite of gap junction formation in hemocytes. Hemocytes from distantly related insects (cockroach and moth) formed functional gap junctions with each other, suggesting sequence homology among gap junction proteins in insects. Using a quantitative dye-transfer assay, it was determined that trypsin reduced the formation of functional gap junctions between hemocytes, but only if the surfaces of both 'dye-donor' and 'dye-recipient' cells were trypsinized. This could be due to cleaving of GJ protein or cell adhesion molecules on the hemocyte surface.

Hemocyte flattening *in vitro* was delayed by plating cells on lipophorin-coated coverslips in order to facilitate patch-clamp studies of these cells. When double whole-cell voltage-clamp was used to measure gap junction formation between cells which were pushed together, electrical coupling was detected within one second of cell-cell contact. Junctional conductance

increased in staircase fashion with steps corresponding to single channel conductances of 345 pS.

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LIST OF ABBREVIATIONS

apo	apolipoprotein
BSA	bovine serum albumin
cDNA	complementary DNA
CNS	central nervous system
d	density
EDTA	ethylenediaminetetra acetic acid (disodium salt)
g	gram
GJ	gap junction
HAI	hemocyte adhesion inhibitor
HDL	high density lipoprotein
IgG	immunoglobulin
LDL	low density lipoprotein
LPS	lipopolysaccharide
M	molar
ml	millilitre
mM	millimolar
min	minute
PAGE	polyacrylamide gel electrophoresis
pS	picosiemens
SDS	sodium dodecyl sulfate
sec	second
SEM	standard error of the mean
VLDL	very low density lipoprotein
µg	microgram
µl	microlitre
µm	micrometre
xg	times gravity
°C	degrees Celcius

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Chapter 1: General Introduction

1.1. Introduction

Insects have evolved effective means of protecting themselves against infection by viruses, bacteria, protozoans, fungi, and helminths. Had they not, they could never have achieved such tremendous success, whether measured in terms of the vast number of insect species and individuals or the colonization by insects of virtually all habitats on Earth.

In addition to being a fascinating branch of cell biology, insect immunology should be studied due to its potential to affect the lives of human beings in three distinct ways. First, insects serve as vectors for organisms responsible for some of the most devastating infectious diseases of humans, such as malaria, sleeping sickness, elephantiasis, and yellow fever (Lehane, 1991). Obtaining a better understanding of insect immune mechanisms and of how certain microorganisms are able to evade and/or suppress these mechanisms (discussed in more detail below) is therefore of great medical importance. For example, a strain of the mosquito, *Anopheles gambiae*, resistant to infection by several species of *Plasmodium* (the protozoan which causes malaria) has been developed by successive selection. This raises the possibility of genetically manipulating natural vector populations as a strategy for controlling malaria (Collins et *al.*, 1986). Second, a clearer understanding of insect immune mechanisms is important from an economic and ecological

standpoint. Due largely to the negative impact of insecticides on humans and on the environment, there has been a move toward the development of ecologically-safer, biological control strategies. Since the control of pest insects by pathogens, such as *Bacillus thuringiensis* (Aronson *et al.*, 1986) and baculoviruses (Wood and Granados, 1991), depend upon successful parasitization of insects, the long-term success of these approaches will depend, in part, on understanding how the immune system of target insects respond to infection by these control agents. Third, studies of invertebrate immunity have recently provided insights into the working of our own immune system. Two classes of antibacterial proteins, the cecropins and defensins, have been found to exist in both insects and vertebrates (Kimbrell, 1991). In addition, recent sequencing of the cDNA coding for the bacteria-inducible insect hemolymph protein, hemolin, has shown that it belongs to the immunoglobulin superfamily (Sun *et al.*, 1990). Regions critical for function might be located by comparing related insect and vertebrate immunoproteins in order to find regions of greatest sequence conservation.

1.2. Insect immunity and wound repair

The overview which follows focuses primarily on the involvement of hemocytes (blood cells) in insect immunity and wound repair. Since humoral components are implicated in most, if not all, aspects of hemocyte behaviour, they are also discussed as necessary.

1.2.1 Mode of entry of a foreign object

The integument, consisting of the cuticle, epidermis (a monolayer of cells which underlies the cuticle it secretes), and basal lamina, serves as an effective barrier preventing the entry of most organisms into the insect hemocoel (body cavity). There are, however, several ways in which organisms gain access to the hemocoel:

(a) through the cuticle

Certain species of fungi produce cuticle-degrading enzymes which enable them to burrow into the hemocoel (Havukkala *et al.*, 1993).

(b) through the intestinal wall

Following ingestion by an insect, acanthocephalans (Mercer and Nicholas, 1967; Robinson and Strickland, 1969), nematodes (Ho *et al.*, 1982; Steinkraus *et al.*, 1993), viruses (Miranpuri *et al.*, 1992; Begon *et al.*, 1993), protozoa (Wernsdorfer, 1980), bacteria (Rahmet-Alla and Rowley, 1989), and fungi (Ferron, 1985), are able penetrate the intestinal wall, thereby gaining access to the hemocoel and internal tissues.

(c) injection (during oviposition)

Endoparasitic wasps inject one or more eggs into the hemocoel of a suitable host (often a lepidopteran larva) where development occurs (Krell, 1991). Polydnavirus co-injected with egg(s) suppress the normal immune response of the host, enabling the parasitoid egg and larva to develop unimpeded.

(d) integumental wound

A break in the integument provides a means of entry to the hemocoel for microorganisms. Insects are especially vulnerable to integumental wounding after a moult, when the newly secreted cuticle has not yet hardened.

In 'c' and 'd' above, and probably in 'a' and 'b' as well, the integument is damaged during entry of the parasite into the hemocoel. Any artificial means of implanting biotic or abiotic objects in the hemocoel (e.g. injection or through an incision in the integument) or of obtaining hemocytes for *in vitro* study also involve the exposure of hemocytes to wounded integument. This is significant since injury has been shown to affect the behaviour of hemocytes both *in vivo* (Rowley and Ratcliffe, 1978; Treherne *et al.*, 1984) and *in vitro* (Cherbas, 1973), and to induce the production of proteins involved in insect immune responses, such as antibacterial proteins and lectins (Komano *et al.*, 1980; Boman *et al.*, 1981; Okada and Natori, 1983; Trenczek and Faye, 1988). It is not at all surprising that such an intimate interrelationship between wound and immune responses should have evolved in insects since a wound site is a likely entry site of infection. That it is not possible to bleed an insect without exposing its hemocytes to putative wound factors should be kept in mind when interpreting results of *in vitro* experiments on supposedly 'unactivated' hemocytes. (There is one report in which it was claimed that a method had been developed for obtaining hemocytes without exposing them to

damaged integument (Cherbas, 1973)).

1.2.2. Hemocytic responses to invasion of the hemocoel

Phagocytosis

The blood system of insects consists of an open hemocoelic space through which the hemolymph (plasma + hemocytes) circulates freely. Small biotic or abiotic objects, when recognized as foreign to the hemocoel, are phagocytosed by hemocytes (Lackie, 1988b). The percentage of insect hemocytes which are phagocytic varies depending upon specific assay conditions and the species being studied (Ratcliffe *et al.*, 1985). Differences in experimental parameters, such as temperature and calcium ion concentration (Brookman *et al.*, 1988), undoubtedly account for some of this variation. However, the consistent finding that 10% of *P. americana* hemocytes were phagocytic in 3 independent studies, two performed *in vivo* (Smith *et al.*, 1986; Lackie and Holt, 1988) and one *in vitro* (Rowley and Ratcliffe, 1980), suggests that at least part of the variation is due to interspecific differences. Neither characteristic morphological features nor distinctive labelling properties (e.g. antibody or lectin binding) have yet been found which distinguish phagocytic from non-phagocytic hemocytes.

Encapsulation

Foreign biotic and abiotic objects too large to be phagocytosed are encapsulated. During encapsulation, hemocytes

adhere and flatten onto an object and one another forming a capsule many hemocyte layers thick (Gotz, 1986; Lackie, 1988b). Capsules typically consist of three layers: an inner layer of necrotic and melanized hemocytes, a middle layer of extremely flattened hemocytes, and an outer layer of rounded, loosely-adherent hemocytes. Maximum recruitment of cells to a capsule is completed by about 24 hours (Lackie *et al.*, 1985), at which point the outermost hemocytes of a capsule may detach and re-enter circulation (Gotz, 1986). Mature capsules become coated with material which has ultrastructural and staining similarities with the subepidermal basal lamina (Lackie *et al.*, 1985). Once this coating has been produced, the capsule seems to be treated as 'self' and presumably remains in the hemocoel for the rest of the insect's life.

Encapsulation is a more complicated process than phagocytosis as it involves not only the adhesion of hemocytes to a foreign surface, but also to one another. This raises an interesting question: what triggers the recruitment of hemocytes to a capsule once the foreign surface has been completely covered by hemocytes? The surface charge of a foreign object has been found to affect the thickness of the capsule formed around it (Lackie, 1983), however the physiological significance of this finding is unclear.

Several types of intercellular junctions form between hemocytes during encapsulation, including gap junctions (GJs) (Baerwald, 1975; Gupta and Han, 1988; Chang *et al.*, 1991), septate junctions (Gupta and Han, 1988), and desmosomes

(Baerwald, 1975; Brehelin *et al.*, 1975; Norton and Vinson, 1977; Schmit and Ratcliffe, 1977; Han and Gupta, 1989). Such extensive interaction between hemocytes during encapsulation emphasizes the importance of a coordinated hemocyte effort. Since hemocytes are normally monodisperse, circulating cells, intercellular junctions form *de novo* during encapsulation. Obtaining a better understanding of hemocyte GJ formation by utilizing an *in vitro* model of encapsulation was one of the aims of my research (see section 1.5).

1.2.3. Wound Healing

Hemocytes are involved in repairing wounds to the integument (Lai-Fook, 1968; Rowley and Ratcliffe, 1978; Lackie, 1988b, figure 4) and internal tissues, such as the central nervous system (Treherne et al., 1984; Howes *et al.*, 1987). Hemolymph coagulation at an integumental wound site occurs within minutes of injury and serves to prevent excessive hemolymph loss (Rowley and Ratcliffe, 1978). Coagulation apparently involves the crosslinking of a hemocyte coagulogen (released during the degranulation of hemocytes near the injury site) with a plasma coagulogen (identified as the plasma lipoprotein, lipophorin) (Barwig, 1985). The stimulus which initiates coagulation has not been identified. Within several hours, large numbers of hemocytes migrate to a wound site, adhere, and flatten, forming an intact multicellular sheath which covers over the wound (Rowley and Ratcliffe, 1978). Epidermal cells subsequently migrate along

this hemocyte sheath, eventually reforming an intact epidermal layer.

In addition to the connection between injury and immune responses mentioned above, wound healing and encapsulation both involve a change in which non-adherent, monodisperse hemocytes in circulation become adhesive to a substrate (foreign object or wound site) and to one another. Thus, during both processes hemocytes construct a functional tissue.

1.2.4. Recognition of Foreignness

Our understanding of how objects are recognized as foreign to the insect hemocoel is fragmentary at best. Using differently charged ion-exchange beads, it has been shown that the net charge of foreign surfaces affects the extent to which objects become encapsulated (Lackie, 1983; Lackie, 1986a). This could be due to the effect of substratum surface charge on adsorption of factors which might be necessary for adhesion to occur (i.e. opsonins), or to a direct effect on cell adhesion itself (Lackie, 1988b). The hydrophobicity of a foreign surface has also been shown to affect capsule thickness (Lackie, 1986b).

Grafting experiments indicate that recognition of foreign tissue implanted in the hemocoel depends to a large extent on the phylogenetic relatedness of the recipient and donor species (Lackie, 1986b). Grafts from phylogenetically distant species are more thickly encapsulated than those from closely related species, whereas allogenic grafts are not encapulated

at all.

Extensive work has been done to try to identify opsonins in insects (i.e. molecules which coat the surface of foreign objects thereby facilitating their recognition and subsequent phagocytosis/encapsulation). Opsonin candidates include:

a) agglutinins

Components with hemagglutinating activity are present in the hemolymph of insects (e.g. Komano *et al.*, 1983; Stebbins and Hapner, 1985; Kubo and Natori, 1987). Most of these agglutinins have been identified as carbohydrate-binding proteins (i.e. lectins), which can be either constitutively present in the hemolymph (Kubo and Natori, 1987) and/or induced upon injury (Komano *et al.*, 1983; Takahashi *et al.*, 1986). While some studies support the proposal that agglutinins play an opsonic role in insects (Drif and Brehelin, 1989; Wheeler *et al.*, 1993), others do not (Rowley and Ratcliffe, 1980; Ratcliffe and Rowley, 1983).

b) lipopolysaccharide-binding protein

Lipopolysaccharides (LPS) are components of the cell wall of Gram-negative bacteria which have been shown to activate cellular defenses in insects (Ratcliffe *et al.*, 1984; Gunnarsson and Lackie, 1985). An LPS-binding protein has recently been purified from the hemolymph of *P. americana* (Jomori *et al.*, 1990) and its cDNA sequenced (Jomori and Natori, 1991). In both *in vivo* and *in vitro* experiments, the

LPS-binding protein was shown to opsonize bacteria (Jomori and Natori, 1992).

c) components of the prophenoloxidase system

The prophenoloxidase system forms part of the immune repertoire of insects (Ashida, 1990) and other arthropods (Johansson and Soderhall, 1989). Activation of the system leads to the production of phenoloxidase, which is responsible for the melanization often observed in response to invasion by foreign objects and during wound healing. The prophenoloxidase system also generates sticky proteins which have been shown to have opsonic activity according to some (Leonard *et al.*, 1985; Brookman *et al.*, 1988), but not all studies (Dularay and Lackie, 1985).

d) hemolin

Constitutively present in low amounts in hemolymph, hemolin (=protein P4) is the major protein induced in lepidopteran larvae and pupae in response to injection of bacteria (Andersson and Steiner, 1987; Ladendorff and Kanost, 1990). Recent sequencing of the cDNA encoding hemolin indicates that it belongs to the immunoglobulin superfamily (Sun *et al.*, 1990; Ladendorff and Kanost, 1991). The ability of hemolin to bind to bacteria suggests that it may be opsonic (Sun *et al.*, 1990; Ladendorff and Kanost, 1991), however this proposal has not been tested.

e) integumental factors

All means of gaining entry to the hemocoel apparently involve damage to the integument or intestinal wall (discussed above). Thus, perhaps with the exception of the eggs of endoparasitic wasps which are injected directly into the hemocoel of their host, foreign objects are exposed to (potentially opsonic) components released from damaged epidermis as they enter the hemocoel.

1.2.5. Humoral antibacterial proteins

The production of bacteriolytic and bacteriostatic proteins in response to wounding and/or infection represents another component of the insect defense repertoire. Several excellent reviews have been published on this subject (Boman and Hultmark, 1987; Kimbrell, 1991; Boman, 1991). The finding that two classes of antibacterial proteins, defensins and cecropins, are found in both insects and mammals (Kimbrell, 1991) emphasizes the fundamental importance of this aspect of immune defense.

1.3 Evasion of Host Defense Reactions

While most organisms entering the hemocoel are recognized as foreign and attacked, some parasites are able to develop within the insect hemocoel without being inactivated by host defense mechanisms. Study of evasion of the immune system by pathogens of insects will undoubtedly provide us with a better understanding of normal immune responses. There are two basic

strategies which are used by parasites, either separately or in combination, to avoid the immune mechanisms of their insect hosts:

(1) passive evasion

Certain parasites passively evade detection by having a surface which is sufficiently similar to the surface of host tissues that it does not elicit an immune response (Lackie, 1976; Davies and Vinson, 1986). Oviposition by endoparasitic wasps directly into the hemocoel of a lepidopteran host may itself represent a form of passive evasion, since it would prevent eggs from being coated by components from damaged epidermis which might be important in immune recognition.

(2) active immunosuppression

Some parasites survive in the hemocoel by suppressing the normal immune response(s) of their hosts. A fascinating example of this involves the complex interaction between parasitic wasps, their symbiotic viruses, and their lepidopteran hosts. Many ichneumonid and braconid hymenopertera carry within their ovaries polydnavirus, so-named because they possess a polydisperse double-stranded DNA genome (Krell, 1991). Stored in the lumun of the ovaries, polydnavirus is co-injected with the parasitoid egg into host caterpillars during oviposition. Polydnaviruses protect the developing parasitoid by inhibiting the phagocytic and adhesive capabilities of host hemocytes (Stoltz and Guzo,

1986; Davies and Vinson, 1988; Strand and Noda, 1991).

1.4. Problems associated with the *in vitro* study of insect immunity

Obtaining a more complete understanding of the factors and steps involved in hemocyte activation depends upon the study of hemocytes *in vitro* under controlled conditions. *In vitro* studies have allowed, for example, for the behaviour of separated fractions of hemocytes to be studied (Mead *et al.*, 1986; Huxham and Lackie, 1988).

A major problem encountered when handling arthropod hemocytes is the tendency of certain hemocytes to degranulate and/or lyse *in vitro* (Lackie et *al.*, 1985). Salines containing EDTA (a calcium chelator) have been found to stablize insect (Mead *et al.*, 1986) and other arthropod hemocytes (Soderhall and Smith, 1983) *in vitro*. However, since many aspects of the insect immune response are calcium-dependent, such as coagulation (Bohn, 1986), prophenoloxidase activation (Leonard *et al.*, 1985), and lectin binding (Richards *et al.*, 1988), the need to incubate hemocytes in calcium-free saline in order to maintain hemocyte stability *in vitro* is undesirable.

A further difficulty relates to the adhesion of hemocytes to substrates *in vitro* in the apparent absence of any activating factor(s). For this reason most studies of phagocytosis have been done using hemocyte monolayers. In many *in vitro* studies of phagocytosis, hemocytes are incubated on glass coverslips, non-adherent hemocytes washed away after a

given period of time, and the phagocytic capabilities of the remaining (i.e. adherent) hemocytes assayed (e.g. Brookman et *al*., 1988; Anggraeni and Ratcliffe, 1991). This experimental system is suboptimal for two reasons: (1) the non-adherent hemocytes which are washed away may have a role to play in phagocytosis, and (2) the hemocytes being studied are adherent and flattened, and therefore are at least partially activated, unlike circulating hemocytes *in vivo*. Clearly, a means of maintaining hemocytes in a non-adhesive state *in vitro* in the presence of calcium would facilitate their study.

1.5. Thesis Objectives

My thesis focuses on two separate, but related, aspects of hemocyte behaviour: (1) the *de novo* formation of gap junctions between *P. americana* hemocytes *in vitro*, and (2) the identification of components (one in *P. americana* plasma and one in vertebrate serum) which inhibit the adhesion of cockroach hemocytes to substrates *in vitro*.

(1) GJs form between hemocytes *in vivo* soon after they are added to a hemocyte capsule (Baerwald, 1975; Han and Gupta, 1989; Chang et *al*., 1991). The demonstration of dye- and electrical-coupling between hemocytes in a 72-hour-old hemocyte capsule confirmed that the the GJs formed during encapsulation are functional (Caveney and Berdan, 1982). Since hemocytes are normally free-floating monodisperse cells *in*

vivo (i.e. they do not have pre-formed GJs), the formation of GJs between hemocytes *in vitro* would provide an excellent system for studying the *de novo* formation of GJs. I therefore decided to address the following questions in my research (Chapter 4):

a) Do hemocytes form GJs *in vitro*? If so, how rapidly do they form?

b) Can hemocytes derived from distantly related insect species form functional GJs in co-culture?

c) Can GJ formation be disrupted by cleaving hemocyte surface proteins?

(2) In the course of experimenting with hemocytes, I was surprised to discover that there is a component in cockroach plasma (and subsequently a component in calf serum) capable of inhibiting the *in vitro* adhesion of hemocytes to glass coverslips. I therefore focused on the following questions (Chapters 2 & 3):

a) What component(s) in insect plasma inhibits hemocyte adhesion *in vitro*?

b) Does vertebrate serum contain similar components capable of inhibiting hemocyte adhesion? If so, where might be sites responsible for inhibiting hemocyte adhesion located on this molecule(s)?

Chapter 2: Lipophorin Inhibits Hemocyte Adhesion *In Vitro*

2.1 Introduction

Insect hemocytes, normally discoid cells circulating in the hemolymph, are capable of adhering and flattening rapidly under certain circumstances. At the site of an injury to the integument (Rowley and Ratcliffe, 1978) or internal tissues, such as the CNS (Treherne *et al*, 1984), hemocytes flatten and aid in wound repair. When a foreign object, either biotic or abiotic, is implanted into the hemocoel, hemocytes adhere and flatten onto it forming a multilayered capsule (Lackie *et al*., 1985; Gotz and Boman, 1985). While it is unclear what triggers hemocyte adhesion in either of these cases, it is apparent that different hemocyte types interact during cellular defense reactions (Ratcliffe *et al*., 1984; Huxham and Lackie, 1988; Anggraeni and Ratcliffe, 1991). The hemocyte types and events involved in the activation of hemocyte adhesion are difficult to identify since hemocytes incubated *in vitro* rapidly adhere to substrates in the apparent absence of any activating factors. An improved ability to maintain hemocytes in a non-adherent, inactivated state *in vitro* would thus be beneficial.

Serum albumin, fibrinogen, and fibronectin have each been shown to inhibit adhesion of vertebrate neutrophils *in vitro* (Bignold *et al*., 1990). In experiments on the annelid *Lumbricus terrestris*, coelomic fluid (=hemolymph) inhibited the adhesion of coelomocytes (=hemocytes) to glass slides *in vitro* (Stein and Cooper, 1981). The effects of insect plasma

components on hemocyte-substrate adhesion, however, have not been investigated until now. In this chapter I show that lipophorin, an abundant, multifunctional lipoprotein found in insect plasma, but not lipophorin-deficient plasma or bovine serum albumin (BSA), can inhibit the adhesion of cockroach (*Periplaneta americana*) hemocytes to glass coverslips *in vitro*, both in the absence and presence of Ca^{++}.

2.2 Materials and Methods

2.2.1 Insects

Adult male and female cockroaches (*Periplaneta americana*) were obtained from a colony reared at 28°C and 65-70% relative humidity with a 12 hour light-12 hour dark photoregime. Food (Dog Chow mixed with refined sugar) and water were supplied *ad libitum*.

2.2.2 Plasma preparation

For experiments in which a specified concentration of plasma was used (see Results: figures 2.1, 2.2, 2.3, & 2.4), 10 μl of hemolymph per cockroach was **quickly** (<0.5 sec) collected (at 26°C) through a puncture at the base of metathoracic leg into a glass micropipet containing 15 μl of Sal I (125 mM NaCl, 13 mM KCl, 17 mM EDTA-2Na^+, 10 mM HEPES, 1 mM $NaHCO_3$, pH to 5.4 or 6.8 with NaOH), and then **immediately** (1 sec) pipetted into 475 μl of this saline. Hemocytes were pelleted at 410 g for 3 min and the supernatant (=2% plasma) pipetted off, diluted with Sal I, and either used immediately or stored frozen at -20°C. In initial experiments (see Results: Fig. 2.1), Sal I at pH 5.4 and 6.8 was tested, however in all subsequent experiments only Sal I (pH 6.8) was used.

For all other experiments, plasma was collected in a refrigerated room at 4°C. Cockroaches (chilled for 30 min) were injected with 200 μl of ice-cold Sal I between abdominal

sternites using a 30-gauge needle. Hemolymph was then collected from a puncture at the base of a metathoracic leg. Hemolymph from 20-30 cockroaches was pooled. Hemocytes were pelleted (610 g for 10 min) and the supernatant (=plasma) filtered (0.22 μM pore size). Plasma was stored frozen at -20°C.

2.2.3 Fractionation of cockroach plasma using Centricon filters

Plasma was fractionated using Centricon filters (Amicon) with molecular weight cutoffs of 10, 30, or 100 kDa as per the Centricon instruction manual. Filtrate (i.e. containing plasma components smaller than the cutoff of the particular Centricon filter) and retentate (i.e. > cutoff) were tested for their ability to inhibit hemocyte adhesion.

2.2.4 Lipophorin preparation

Lipophorin was isolated from plasma by single-spin density gradient ultracentrifugation essentially according to Shapiro *et al.* (1984). KBr (1.32 g) was dissolved in 3 ml of plasma, and 3.2 ml of this KBr/plasma mixture was overlayed with 3.2 ml of 0.9% NaCl in a 14 x 89 mm Beckman polyallomer ultracentrifuge tube (mineral oil was used to top-up the tubes). Tubes were placed into a Beckman SW41Ti rotor and centrifuged at 37,000 rpm (=235,000 g at R_{max}) for 4 hours at 10°C in a Beckman L8M Ultracentrifuge. A tube lacking plasma was centrifuged with the sample tubes, fractionated, the

refractive index read, and the densities calculated from the refractive index of KBr at 25°C.

Following centrifugation most of the yellow band (lipophorin) was removed through a hole pierced in the side of the tube. The bottom of the tube was then punctured and the fluid beneath the remainder of the yellow band collected (lipophorin-deficient plasma). KBr was removed from the samples by centrifuging them in Centricon 10s (i.e. 10 kDa cutoff) at 4300 g for 120 min at 10°C, adding Sal I to the retentates to bring each to 2 ml, repeating the centrifugation step, and then adding Sal II (150 mM NaCl, 13 mM KCl, 10 mM HEPES, 1 mM $NaHCO_3$, pH to 6.8 with NaOH) to the retentates to bring each to 1 ml. Aliquots of the protein samples were stored at -20°C.

2.2.5 Adhesion assay

Cockroaches were punctured at the base of a mesothoracic leg, 4 µl of hemolymph was quickly collected into a glass micropipet containing 10 µl of Sal I, and the mixture immediately pipetted into a tube containing 500 µl of this saline. Hemocytes were pelleted (370 g for 1 min), resuspended in 500 µl of test solution, and pipetted onto a coverslip apparatus (consisting of a Teflon O-ring clamped onto a glass coverslip by a metal plate). Test solutions were made up in either Sal I or Perisal (= Sal II + 2 mM $CaCl_2$-$2H_2O$). Thirty minutes after plating the cells, the coverslips were rinsed 6 times with 500 µl Sal I. Prior to and following rinsing, 10

fields of view were selected at random and videotaped (20x objective lens) or photographed (40x objective lens) using a Zeiss Axiovert 35 Inverted Microscope with phase contrast optics. During videotape playback hemocytes in the 10 fields of view (before and after rinsing) were counted and the percentage of hemocytes adhering determined.

To verify that the cells that failed to adhere in plasma were not damaged or dead, hemocytes were incubated in 1% plasma (in Sal I) for 30 minutes, non-adherent cells pipetted from the coverslip and pelleted. The cells were then resuspended in fresh Sal I, pipetted onto a new coverslip, rinsed 6 times after 30 minutes, and the percentage of adhering determined as before. Each adhesion assay was repeated three or more times and the standard error of the mean determined.

2.2.6 Cleaning of coverslips and O-rings

Glass coverslips (24 x 20 mm) were soaked in 95% ethanol, then in endotoxin-free sterile water (Abbott Labs), and autoclaved. Teflon O-rings (14 mm diameter) were soaked in Alconox detergent, rinsed, boiled in sterile water, and autoclaved. Sterile water was used in the preparation of all salines.

2.2.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis of proteins was performed according to the method of Laemmli (1970). Gel concentrations for

separating gel and stacking gel were 5 and 4%, respectively. The samples were heated to 100°C for 3 minutes in 3-4 volumes of sample buffer (containing either 5% or 10% mercaptoethanol) before being loaded onto the gel. For the determination of molecular weight, rabbit muscle myosin (205 kDa), *E. coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase b (97.4 kDa), and bovine serum albumin (66 kDa) were used as standards (Sigma).

2.2.8 Protein measurement

Protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

2.3 Results

The relatively large hemolymph volume and high concentration of hemocytes in *P. americana* hemolymph (Wheeler, 1963; Lackie *et al.*, 1985), coupled with the ease of rearing this insect in large numbers, make it an excellent species to use for the *in vitro* study of hemocytes. Furthermore, since several studies on the morphology (Baerwald, 1970; Ratcliffe and Price, 1974; Lackie *et al.*, 1985; Chain *et al.*, 1992) and function (e.g. Baerwald and Boush, 1971; Takle and Lackie, 1986) of *P. americana* hemocytes, and on *P. americana* plasma proteins (Chino *et al.*, 1981; Lackie, 1981; Jomori and Natori, 1992) have been published, there is already a substantial body of knowledge specific to this insect.

There are, however, significant technical problems to be overcome when attempting to study *P. americana* hemocytes. Obtaining hemocytes in a monodisperse state for *in vitro* studies is difficult since the hemocytes form large aggregates very rapidly upon bleeding (i.e. <1 second). If hemocyte agglutination is to be avoided, hemolymph must be drawn up into a micropipet preloaded with saline immediately after the integument is punctured and then immediately diluted in a larger volume of saline (Wheeler, 1963; and personal observation). Hemocyte agglutination can also be minimized by bleeding pre-cooled (i.e. to immobility) cockroaches at 4°C, however this results in a change in the proportions of various hemocyte types obtained in bled hemolymph (Lackie *et al.*,

1985). Another problem encountered when handling arthropod hemocytes is the tendency of certain hemocytes to degranulate and/or lyse *in vitro* (Lackie *et al.*, 1985). Salines containing EDTA (to chelate free Ca++) and with low pH have been found to stabilize insect (Mead *et al.*, 1986) and other arthropod hemocytes (Soderhall and Smith, 1983) *in vitro*.

In order to make possible *in vitro* experiments which would require centrifuging and resuspending hemocytes several times, I tried to develop a saline in which hemocytes obtained from adult female cockroaches would retain their *in vivo* charact -istics *in vitro* (i.e. retain granules, and remain discoid and non-adherent (both to each other and to other surfaces)). I observed that when freshly-bled hemolymph (hemocytes + plasma) was pipetted into Sal I (pH 5.4), centrifuged, the supernatant discarded, and the hemocyte pellet resuspended in fresh Sal I (pH 5.4), 85% of the cells adhered to glass coverslips within 30 minutes (Fig. 2.1). However, when bled into Sal I (pH 5.4), centrifuged, and resuspended in the supernatant (containing 1% plasma in Sal I (pH 5.4)), hemocytes did not adhere to coverslips (Fig. 2.1). These results suggested that there was at least one constitutive component in plasma capable of inhibiting hemocyte-substrate adhesion *in vitro*. The identification of this hemocyte adhesion inhibitor (HAI) became my goal.

Two modifications were then made to this experimental system prior to characterization and purification of the HAI: (1) The pH of *P. americana* plasma has been estimated to be

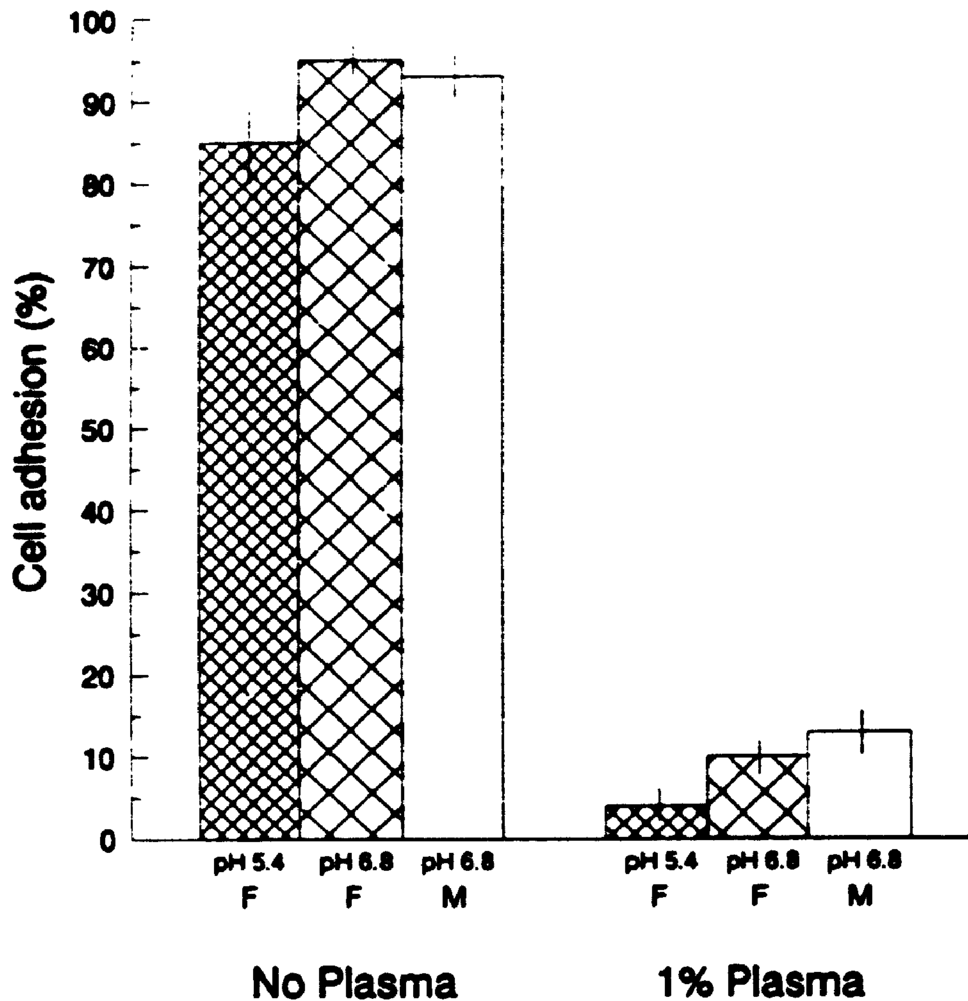


Figure 2.1 Cockroach plasma inhibits the adhesion of cockroach hemocytes to glass coverslips *in vitro*. In the absence of *P. americana* plasma, >85% of hemocytes from either female (F) (in Sal I at pH 5.4 or 6.8) or male (M) P. americana (in Sal I at pH 6.8) adhere, but in the presence of 1% plasma most hemocytes remain non-adherent after 30 min. Bars represent the mean ±SEM for 3 or more separate experiments.

7.17 (Weidler and Sieck, 1977). Since results comparable to those obtained with Sal I (pH 5.4) were obtained when Sal I with the more physiological pH of 6.8 was used (Fig. 2.1), Sal I (pH 6.8) was used in all subsequent adhesion experiments. (Sal I (pH 6.8) is hereafter referred to simply as Sal I.)
2) It was evident that purification of the HAI from adult male *P. americana* plasma would be easier due to the presence of a major plasma protein, vitellogenin, in female, but not male, plasma (Kim *et al.*, 1992). Since HAI activity was detected in both adult male and adult female plasma (Fig. 2.1), adult males were used in all subsequent experiments.

2.3.1 Initial characterisation of the HAI found in cockroach plasma

Concentration-dependence of HAI activity in plasma

Cockroach plasma diluted to 0.1% inhibited hemocyte adhesion by approximately 50%, but did not inhibit adhesion over control levels at 0.04% (Fig. 2.2). Of the hemocytes which had not adhered in the presence of 1% plasma, 95% subsequently adhered when incubated in Sal I in the absence of plasma for 30 minutes (on a fresh coverslip), confirming that the lack of cell adhesion in plasma was not due to damage to the cells.

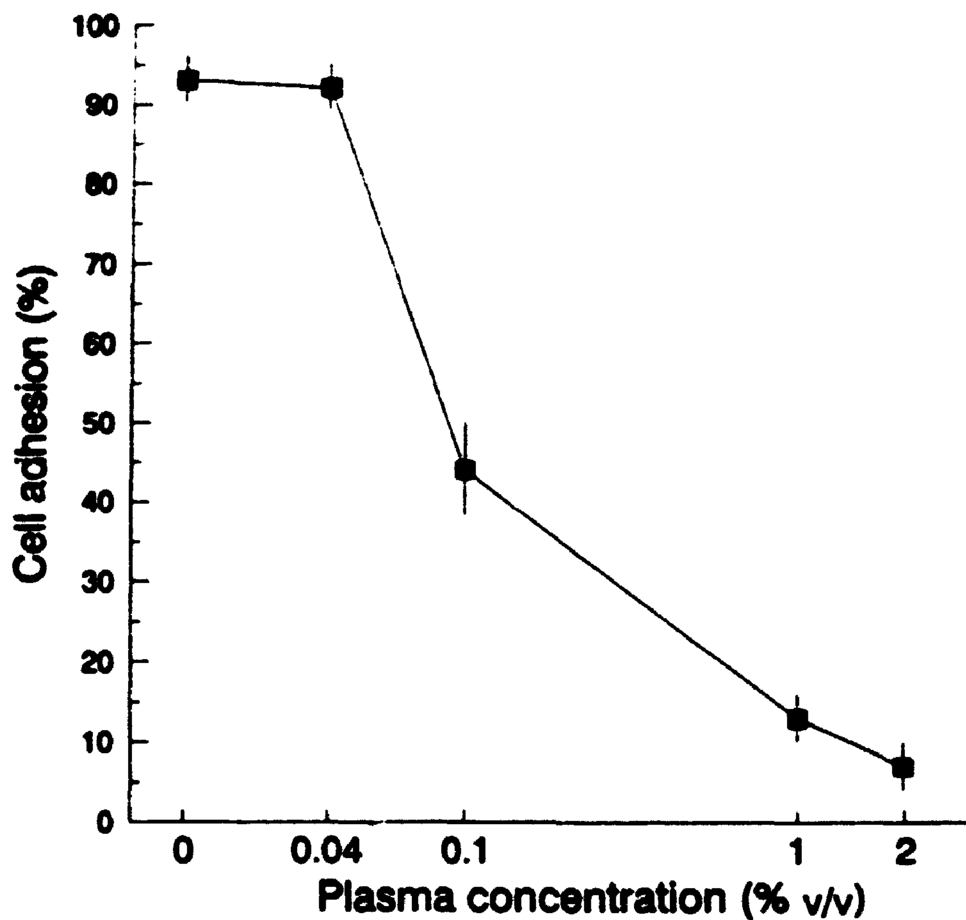


Figure 2.2 Concentration-dependence of plasma on the inhibition of hemocyte adhesion *in vitro* in Sal I (i.e. in the absence of Ca^{2+}). Hemocyte adhesion is inhibited by approximately 50% by 0.1% plasma. Data points represent the mean ±SEM for 3 or more separate experiments.

Heat & Cold Stability of the HAI

The finding that freezing (-20°C) plasma left the activity of the HAI intact (Fig. 2.3) facilitated the purification of the HAI since it meant that cockroach plasma and plasma fractions could be stored frozen. Heating plasma to 70°C for 10 minutes also did not destroy HAI activity, however plasma no longer inhibited hemocyte adhesion when it had been heated to 100°C for 5 minutes (Fig. 2.3).

Estimation of the native molecular weight of the HAI

Centricon filters with cutoffs of approximately 10, 30, and 100 kDa were used to fractionate cockroach plasma and thereby obtain an estimate of the native molecular weight of the HAI. The results of these experiments were consistent with the HAI having a native molecular weight greater than 100 kDa (Fig. 2.4).

2.3.2 The HAI is lipophorin

Lipophorin, a lipoprotein which comprises approximately 50% of the protein in *P. americana* plasma, seemed a good candidate for the HAI since its native molecular weight of approximately 600 kDa (Chino *et al.*, 1981) exceeds the minimum molecular weight for the HAI (100 kDa)(Fig. 2.4). As a simple method for the purification of lipophorin from insect plasma had been published (Shapiro *et al.*, 1984), testing of this

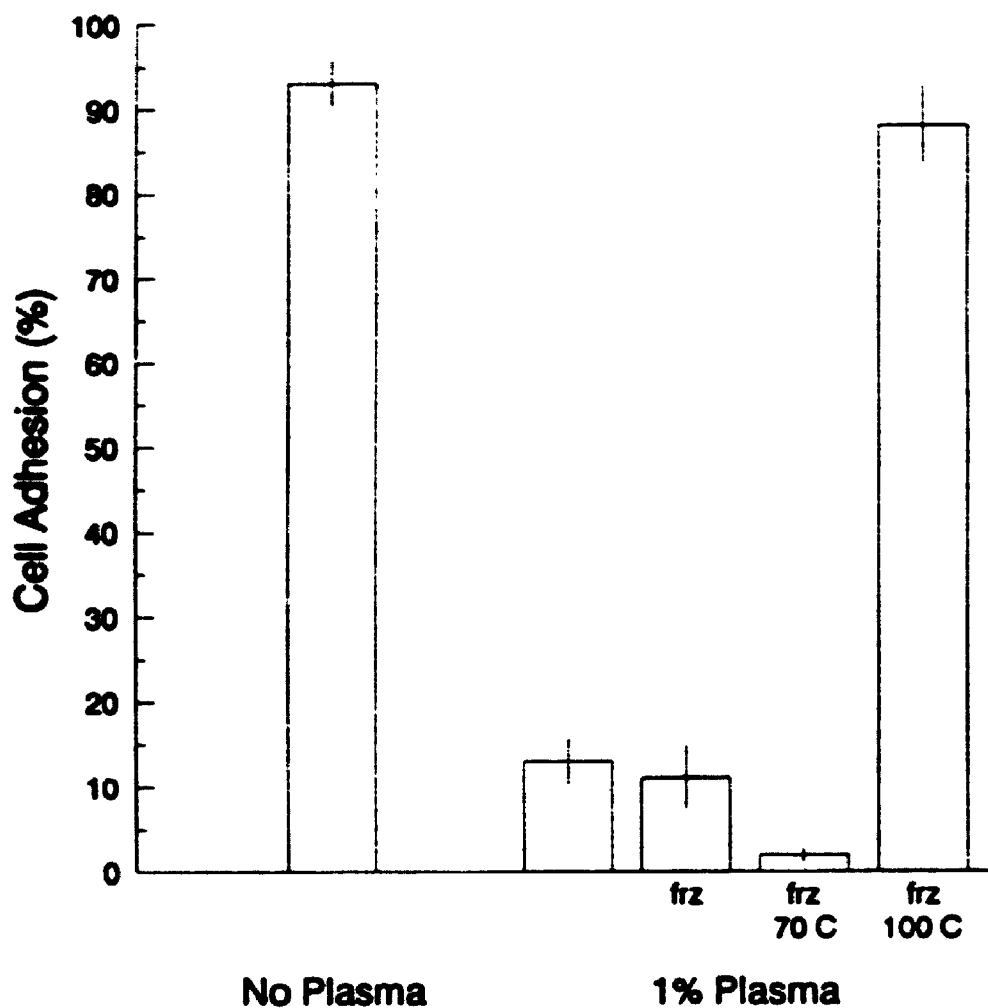


Figure 2.3 Heat and cold stability of the HAI. Whereas neither freezing plasma to -20°C (frz) nor heating it to 70°C for 10 min destroys its ability to inhibit hemocyte adhesion, heating plasma to 100°C for 5 min removes this activity. That heating to 100°C, but not to 70°C for 10 min, resulted in the precipitation of virtually all the protein in plasma suggests that the HAI is a protein. Bars represent the mean ±SEM for 3 or more separate experiments.

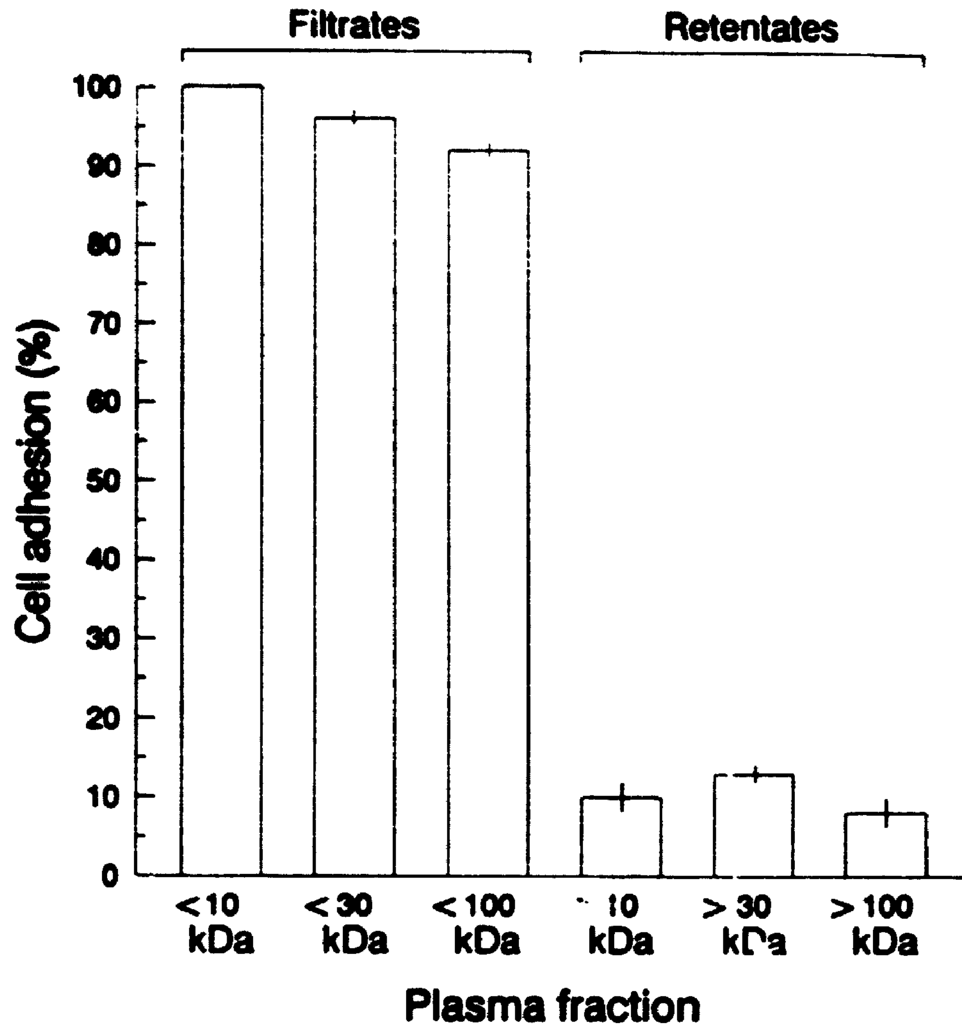


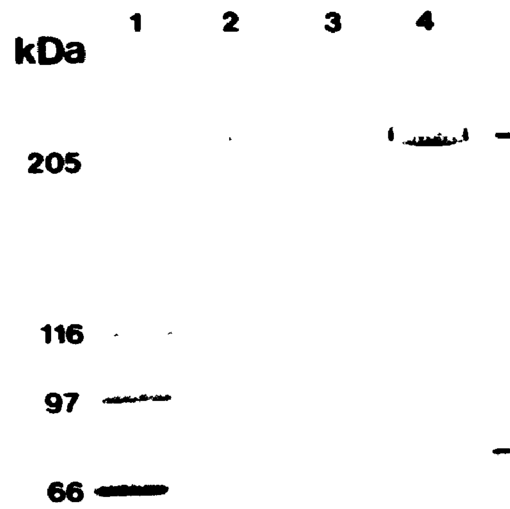
Figure 2.4 Molecular size of the HAI factor. Plasma was filtered using Centricon filters with molecular size cutoffs of 10, 30, and 100 kDa. The presence of hemocyte adhesion inhibition activity only in the retentates indicates that the hemocyte adhesion inhibitor(s) has a native molecular weight >100 kDa. Bars represent the mean ±SEM for 3 or more separate experiments.

hypothesis was rather straightforward.

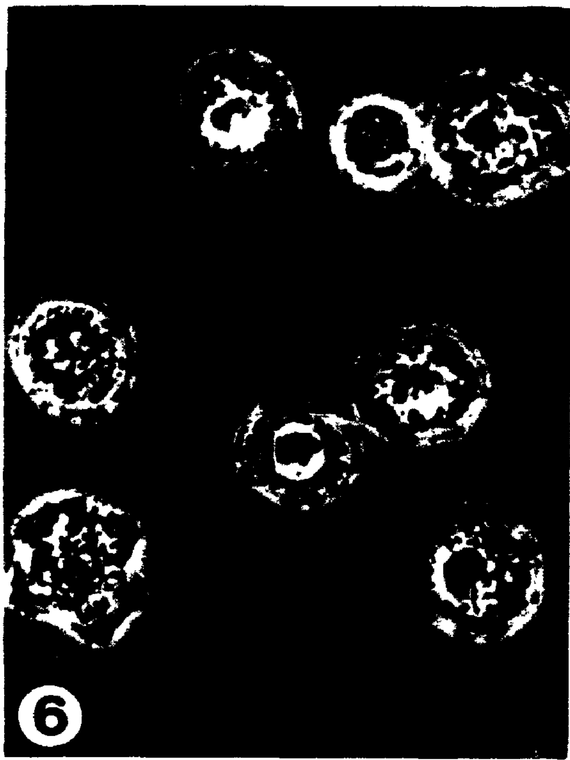
The density of lipophorin purified from *P. americana* plasma by KBr density gradient ultracentrifugation was estimated to be 1.11 g/ml, which is in close agreement with the published values of 1.12 g/ml (Ryan *et al.*, 1984) and 1.10 g/ml (de Kort and Koopmanschap, 1987). Lipophorin consists of 240 and 80 kDa subunits (corresponding to apolipophorin I and II, respectively) when electrophoresed on a 5% SDS polyacrylamide gel (Fig. 2.5). The minor band in the lipophorin lane at 225 kDa has previously been shown to be a degradation product of apoLp I (Ryan *et al.*, 1984). In the course of running lipophorin samples on SDS polyacrylamide gels I found that doubling the concentration of beta-mercaptoethanol in the sample buffer caused most of the apoLp I subunit to stick in the stacking gel (not shown).

The effects of lipophorin and lipophorin-deficient plasma on hemocyte morphology were assessed. In saline, either in the absence (Fig. 2.6) or presence of 2 mM $CaCl_2$ (Fig. 2.7), hemocytes adhered and flattened onto glass coverslips within 30 minutes without any apparent degranulation. However, most hemocytes incubated in 50 μg lipophorin/ ml Sal I for 30 minutes retained the same discoid appearance that they had just after being bled (Fig. 2.8), with only a small fraction of the cells adhering to coverslips (Fig. 2.9). Hemocytes incubated for 30 minutes in Sal I plus either lipophorin-deficient plasma (Figs. 2.10 & 2.11) or BSA (not shown) at 50 μg/ ml adhered and flattened, appearing indistinguishable from

Figure 2.5 SDS-polyacrylamide gel electrophoresis of *P. americana* plasma fractions separated by KBr density ultracentrifugation. Molecular weight markers (lane 1), plasma (8 μg)(lane 2), lipophorin-deficient plasma (5μg)(lane3), and lipophorin (8μg)(lane 4). The positions of the two subunits of lipophorin, apolipophorin I (240 kDa) and II (80 kDa), are marked. The 76 kDa plasma protein seen in lanes 2 and 3 is distinct from apolipophorin II. Proteins in the gel were stained with Coomassie blue.


kDa
1
2
3
4
205
116
97
66

Figures 2.6 & 2.7 Adhesion of *P. americana* hemocytes in vitro. Plasma components were washed away, hemocytes resuspended in either Sal I (containing 17 mM EDTA)(Figure 2.6) or in Perisal (containing 2 mM Ca^{2+})(Figure 2.7), and photographed 30 min after bleeding (prior to rinsing). Scale bar = 20 μm.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Figures 2.8-2.11 Lipophorin inhibits the adhesion of hemocytes in the absence of Ca^{++} *in vitro*. Hemocytes resuspended in saline containing either lipophorin (Figure 2.8) or lipophorin-deficient plasma (Figure 2.10) were allowed to settle onto coverslips. The cells are shown just before (Figures 2.8 & 2.10) and after (Figure 2.9 & 2.11) the coverslips were rinsed at 30 min to remove non-adherent cells. Few cells incubated in lipophorin adhered (Figure 2.9), whereas lipophorin-deficient plasma (Figure 2.11) did not inhibit cell adhesion. Scale bar = 20 μm.

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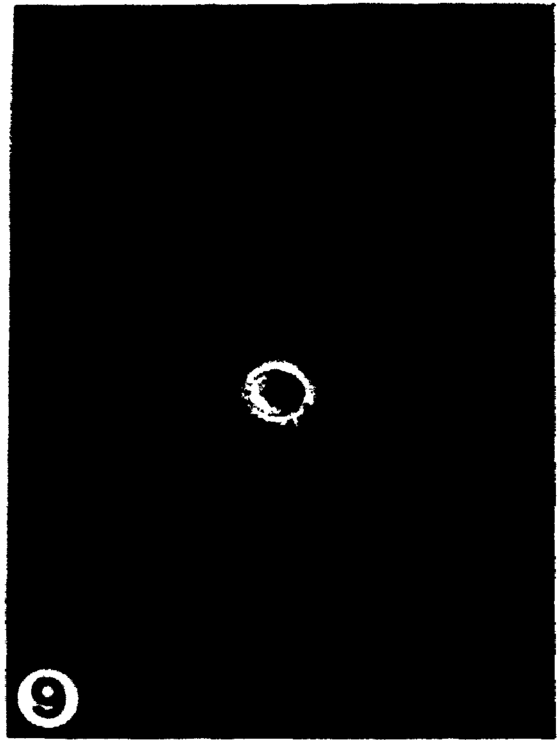
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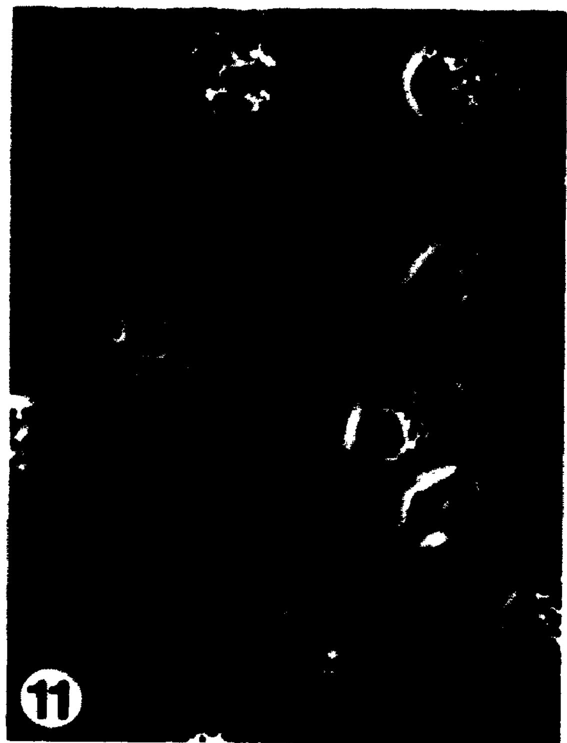
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cells incubated in Sal I alone.

When incubated in 50 μg lipophorin/ ml SAL II + 2 mM Ca^{++} for 30 minutes most hemocytes retained a discoid, nonadherent morphology (Figs. 2.12 & 2.13); however, they sent out more pseudopodia than did cells in the same concentration of lipophorin in the absence of Ca^{++}. In saline containing 2 mM $CaCl_2$, neither lipophorin-deficient plasma (Figs. 2.14 & 2.15) nor BSA (not shown) at a concentration of 50 μg/ ml inhibited cell adhesion and flattening.

The ability of lipophorin, lipophorin-deficient plasma and BSA (at concentrations of 1, 5, 20, and 50 μg/ ml) to inhibit hemocyte adhesion to glass coverslips was tested in the presence or absence of Ca^{++}. Lipophorin inhibited the adhesion of hemocytes both in the absence of Ca^{++} (Fig. 2.16A) and in the presence of 2 mM Ca^{++} (Fig. 2.16B), but lipophorin-deficient plasma was unable to inhibit hemocyte adhesion in either case. BSA, used as a control for non-specific protein effects, also did not block hemocyte adhesion.

Figures 2.12-2.15 Lipophorin inhibits hemocyte adhesion *in vitro* in the presence of Ca^{2+}. Cells were incubated for 30 min in Perisal (i.e. containing 2 mM Ca^{2+}) and either lipophorin (Figure 2.12) or lipophorin-deficient plasma (Figure 2.14), and the coverslips then rinsed to remove non-adherent cells (Figures 2.13 & 2.15), respectively). Scale bar = 20 µm.

12

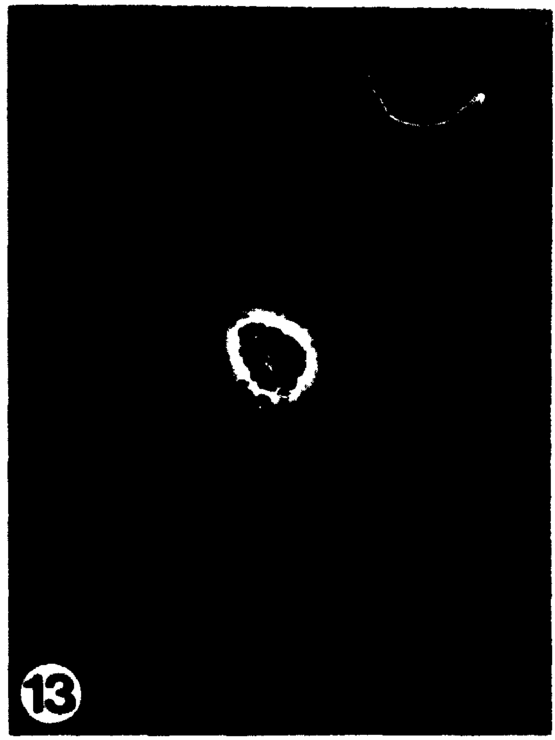
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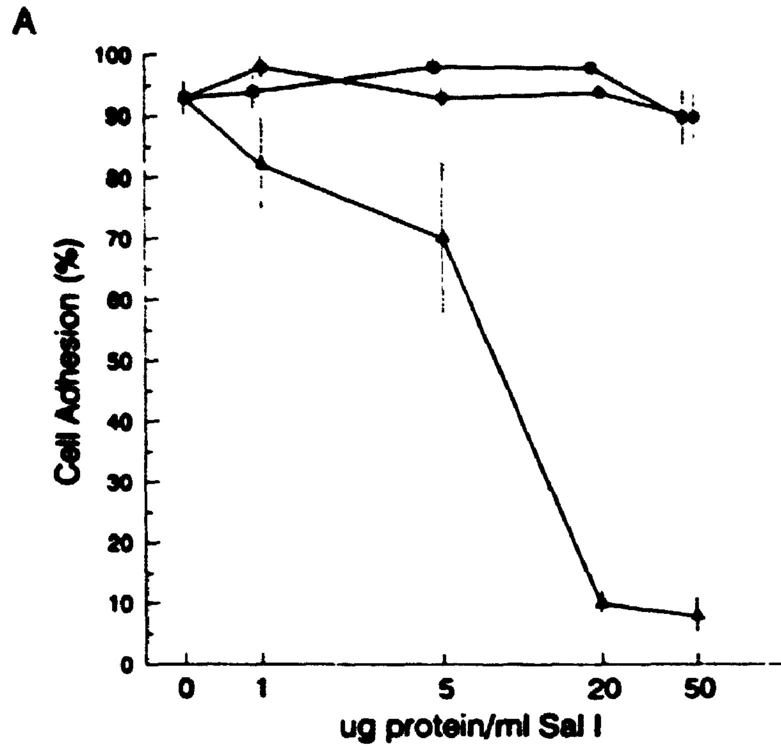
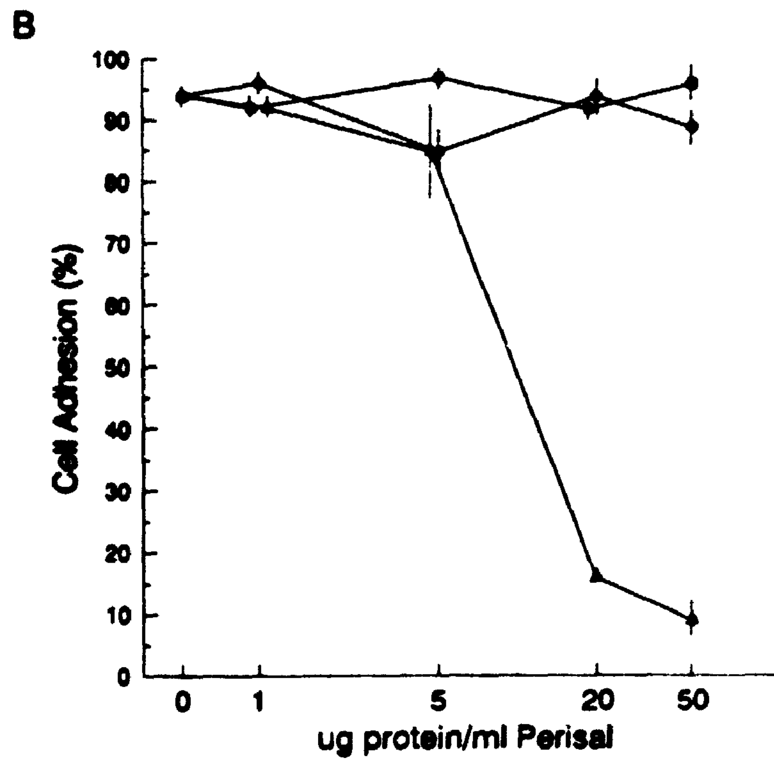
Figure 2.16 Concentration-dependence of lipophorin on the inhibition of hemocyte adhesion. Adhesion of hemocytes *in vitro* is inhibited by lipophorin (triangles), but not by lipophorin deficient plasma (squares) or bovine serum albumin (circles) in either Sal I (containing 17 mM EDTA)[Figure 2.16A] or in Perisal (containing 2 mM Ca^{2+})[Figure 2.16B]. Data points represent the mean ±SEM for 3 or more separate experiments.


A
Cell Adhesion (%)
100
90
80
70
60
50
40
30
20
10
0
0
1
5
20
50
ug protein/ml Sal I


B
Cell Adhesion (%)
100
90
80
70
60
50
40
30
20
10
0
0
1
5
20
50
ug protein/ml Perisal

2.4 Discussion

Lipophorin is a multifunctional lipoprotein which comprises approximately 50% of the total plasma protein in *P. americana* (Chino *et al.*, 1981). Produced in the fat body (Prasad *et al.*, 1986), lipophorin is: 1/ a transporter of lipid, primarily diacylglycerol, in the hemolymph (Shapiro *et al.*, 1988), 2/ a transporter of juvenile hormone in the hemolymph of some insect species (de Kort and Koopmanschap, 1987), 3/ taken up into vitellogenic oocytes (Kulakosky and Telfer, 1990), 4/ involved in clot formation (Brehelin, 1986; Bohn, 1986). In this study I discovered that lipophorin also can inhibit the adhesion of hemccyte-substrate adhesion *in vitro*, even in the presence of Ca^{++}. This finding is important since it should enable several experiments to be carried out on hemocytes *in vitro* which had formerly not been possible as outlined in 2.4.1 below. Perhaps more importantly, inhibition of hemocyte adhesion by lipophorin may also be a feature of the normal regulation of hemocyte activation *in vivo*, by mechanism(s) described in 2.4.2.

2.4.1 Lipophorin as a tool for studying hemocytes *in vitro*

Hemocyte classification and separation

An improved ability to maintain insect hemocytes in an 'inactivated' state *in vitro* may assist in the classification

of hemocytes, an area of considerable controversy. Several insect hemocyte classification schemes have been put forward (Rowley and Ratcliffe, 1981; Brehelin and Zachary, 1986; Gupta, 1991), but there is a lack of consensus as to which should be adopted. Since hemocyte appearance in the light and/or electron microscope has been an important criterion used in the classification of hemocytes, the knowledge that hemocyte morphology *in vitro* is strongly affected by lipophorin may help explain certain discrepancies and aid in standardizing hemocyte typing.

There is growing evidence indicating that different hemocyte types interact during cellular defense reactions in insects (Ratcliffe *et al.*, 1984; Huxham and Lackie, 1988; Anggraeni and Ratcliffe, 1991). To expand our understanding of these interactions it is necessary to separate and maintain pure hemocyte populations *in vitro*. Mead *et al.* (1986) were able to obtain relatively pure hemocyte populations by separating cells on Percoll density gradients. A major problem they encountered, however, was that many cells were lost during handling, especially during the final washes of the cells, despite the use of silicon-coated glassware. Although it remains to be shown that lipophorin can inhibit adhesion of hemocytes from insects other than *P. americana*, it is probable that the low recovery of hemocytes following the washing of separated hemocytes is due to the removal of lipophorin. Using Percoll gradients to separate hemocytes, it may be possible to greatly increase the final hemocyte yields simply by ensuring

that lipophorin is always present.

Several investigators studying hemocytes have been very careful to clean glassware thoroughly prior to use and have prepared salines using endotoxin-free water to prevent hemocyte activation, presumably by small amounts of contaminating endotoxin (Mead *et al.*, 1986; Johansson and Soderhall, 1988; Anggraeni and Ratcliffe, 1991). Although I used sterile endotoxin-free water and autoclaved teflon O-rings and glass coverslips in all adhesion assays, I did not determine whether these measures were actually necessary to prevent hemocyte adhesion in the presence of purified lipophorin.

Importance of studying hemocytes in Ca^{++}-containing salines

Hemocyte behaviour should ideally be studied in the presence of Ca^{++} since several aspects of the insect wound and immune responses are Ca^{++}-dependent:

(1) two steps involved in coagulation, the initial degranulation/rupture of certain hemocytes and the crosslinking of lipophorin to the hemocyte coagulogen (Bohn, 1986)

(2) prophenoloxidase activation (Leonard *et al.*, 1985)

(3) agglutination (Stebbins and Hapner, 1985; Richards *et al.*, 1988) and opsonization (Wheeler *et al.*, 1993) by insect serum lectins

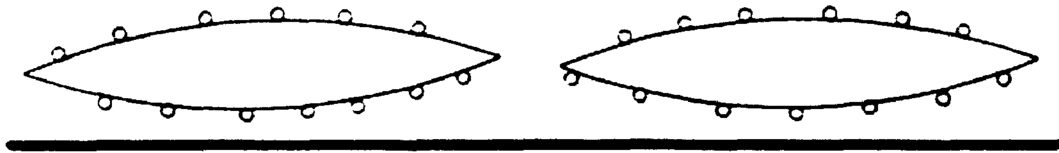
(4) hemocyte binding to a 76 kDa cell adhesion factor (CAF) (Johansson and Soderhall, 1988; Rantamaki *et al.*, 1991)

(discussed below).

However, due to the instability of certain hemocytes in the presence of Ca^{++}, and because coagulation occurs rapidly unless hemolymph is placed immediately into an anticoagulant (Mead *et al.*, 1986; Bohn, 1986), Ca^{++}-chelators such as EDTA are routinely used in hemocyte salines. Using purified lipophorin to stabilize hemocytes *in vitro*, it should now be possible to study Ca^{++}-dependent aspects of the insect immune response on 'non-activated', separated or unseparated hemocytes under physiological conditions (i.e. in the presence of Ca^{++}). Of particular interest will be an examination of the effects of wound factor(s) (Cherbas, 1973), immune response activators (e.g. lipopolysaccharide, peptidoglycan, laminarin), and an encapsulation promoting factor (Davies *et al.*, 1988) on hemocyte behaviour *in vitro*.

2.4.2 Mode of hemocyte adhesion inhibition by lipophorin

I can envision two models to explain how lipophorin inhibits hemocyte adhesion *in vitro* (Fig. 2.17). In one model (Fig. 2.17A), the inhibition of hemocyte adhesion involves the binding of lipophorin to the hemocyte surface. The unloading of lipid from lipophorin at the surface of insect flight muscle has been shown immunocytochemically to be an extracellular event (Van Antwerpen *et al.*, 1988). Subsequent ligand blotting experiments demonstrated that lipophorin bound specifically to a 30 kDa protein in the plasma membrane of locust flight muscle (Van Antwerpen *et al.*, 1990). A



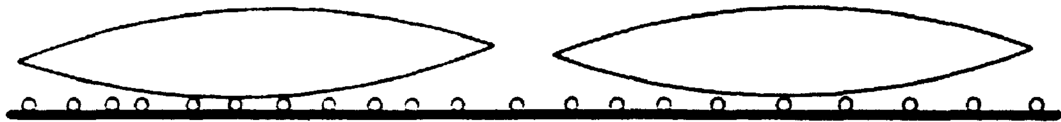


Figure 2.17 Two models for the mode of inhibition of hemocyte adhesion by lipophorin. (A) Lipophorin (small circles) might inhibit adhesion by coating the hemocyte surface or binding to a lipophorin receptor in the plasma membrane. This implies that under 'resting' conditions *in vivo* lipophorin maintains hemocytes in an inactive state. (B) Alternatively, hemocytes might become activated by exposure to a foreign surface (such as glass) unless it is coated with lipophorin.

lipophorin receptor has also been found on fat body membrane (Van Anntwerpen *et al.*, 1989; Tsuchida and Wells, 1990) which has a requirement for Ca^{++} and is inhibited by suramin (a polysulfated polycyclic hydrocarbon shown to inhibit the binding of several lipoproteins to their receptors (George et *al.*, 1987)).

Whereas the lipophorin receptors on fat body and muscle participate in the loading/unloading of lipid from lipophorin, regulation of hemocyte adhesion might involve a lipophorin receptor on the surface of hemocytes which, when occupied, would keep hemocytes in a 'resting' state. This model does not preclude the possibility that other components (e.g. wound factors) might override the stabilizing influence of lipophorin (i.e. hemocyte activation might also occur by other pathways). The fact that neither the absence of Ca^{++} nor the presence of suramin (personal observation) affected the ability of lipophorin to inhibit hemocyte adhesion *in vitro* suggests that if a lipophorin receptor does exist on hemocytes, it has different properties from the receptor found in fat body.

The second model proposes that lipophorin inhibits hemocyte adhesion not by binding to the hemocyte surface, but rather by coating the glass and/or soluble 'contaminants' so that they are not recognized as foreign by hemocytes (Fig. 2.17B). According to this model, hemocytes remain in a 'resting' state unless exposed to activating factors and/or foreign surfaces, the implication being that the inhibition of

in vitro hemocyte-substrate adhesion by lipophorin is an experimental artifact.

Insect organs (e.g. epidermis, fat body, nervous system) are covered by a layer of extracellular matrix (ECM) which serves to separate them from the hemocoel (Ashhurst, 1985). Circulating hemocytes come in contact with this layer but do not normally adhere to it. Is this because the ECM is recognized by hemocytes as 'self', or is the presence of lipophorin in the hemolymph (and/or coating the ECM) needed to keep hemocytes in a 'resting' state? One way to approach this question would be to study hemocyte adhesion *in vitro* on substrates which are similar to those encountered by circulating hemocytes *in vivo*. Glass coated with ECM components (e.g. collagen) or the basal side (i.e. basal lamina) of dissected integumental squares could be used as test substrates. Will hemocytes adhere to 'physiological' substrates such as these, and if so, can hemocyte adhesion be inhibited by lipophorin? (If integument is used, it might prove necessary to use newly moulted insects since the cuticle is more transparent at this stage.)

It is interesting that fibrinogen, one of the vertebrate plasma proteins which inhibits neutrophil adhesion *in vitro* (Bignold *et al.*, 1990), and the insect plasma protein, lipophorin, are both proteins which are incorporated into coagula during a wound response. In the case of insects, this may be significant for understanding why hemocytes adhere at a wound site (Rowley and Ratcliffe, 1978). It is possible that

the incorporation of lipophorin into a coagulum may result in a locally low concentration of free lipophorin, and that this may trigger hemocytes at a wound site to become adhesive. It is difficult to envision, however, how this alone could account for hemocyte binding onto the surface of a foreign object during encapsulation since presumably the concentration of free lipophorin is not diminished in the region surrounding a foreign object.

A 76 kDa cell adhesion factor (CAF) involved in crayfish hemocyte adhesion has been purified from crayfish granular hemocytes (Johansson and Soderhall, 1988), and a 90 kDa protein with similar functional and immunological properties has been isolated from cockroach (*Blaberus craniifer*) hemocytes (Rantamaki *et al.*, 1991). CAF is presumably stored in secretory granules of granular hemocytes in an inactive form, released in response to a stimulus, and activated outside cells to mediate hemocyte attachment and spreading (Johansson and Soderhall, 1988). It is unclear whether adhesion of *P. americana* hemocytes involves a similar protein, since adhesion to CAF is Ca^{++}-dependent while in the present experiments *P. americana* hemocytes were able to adhere in the absence of Ca^{++} as well. It is possible that *P. americana* hemocytes are capable of adhering to a substrate by more than one mechanism, at least one of which is Ca^{++}-independent.

In the next chapter I discuss the discovery that the human lipoprotein, apoB, also inhibits cockroach hemocyte-substrate adhesion *in vitro*. This lends further support to the

notion that the insect protein, lipophorin, and the vertebrate protein, apoB, are derived from a common ancestral protein.

Chapter 3: ApoB-100 Inhibits Hemocyte Adhesion *In Vitro*

3.1 Introduction

Having established that the insect plasma protein, lipophorin, is capable of inhibiting cockroach hemocyte adhesion *in vitro*, I was interested in testing vertebrate serum for the presence of this same activity for several reasons. First, vertebrate serum proteins, including fibronectin and albumin, have been shown to inhibit vertebrate neutrophil-substrate adhesion *in vitro* (Bignold *et al.*, 1990). Although bovine serum albumin failed to inhibit hemocyte adhesion *in vitro*, other vertebrate serum components might be active. Second, calf serum, which is inexpensive and readily-available, contains large quantities of lipoproteins, and therefore can be used to test whether inhibition of hemocyte adhesion is a general property of lipoproteins. Third, calf serum was found to stabilize fragile hemocytes and prevent degranulation *in vitro* (Huxham and Lackie, 1986; Takle and Lackie, 1986; Huxham and Lackie, 1988). It was not determined whether serum affected hemocyte adhesion as it was only added after hemocytes had already adhered.

I show here that calf serum is able to inhibit hemocyte adhesion and that this is due to the presence of serum lipoproteins. The availability (both commercially and from other laboratories) of purified vertebrate lipoproteins, apolipoproteins, and synthetic apoB peptides made it possible not only to determine which lipoprotein was able to inhibit

hemocyte adhesion, but also to locate regions on this molecule involved in inhibiting hemocyte adhesion.

3.2 Materials and Methods

The hemocyte adhesion assay was performed as described in section 2.2.5, with the following modifications:

(1) Six fields of view (instead of 10) were videotaped before and after rinsing the coverslips.

(2) During the one minute centrifugation step, 250 µl of test solutions were pipetted onto each of three coverslip dishes. Following centrifugation, hemocytes were resuspended in 800 µl of saline (instead of 500 µl), and 250 µl of this was added to each of the three coverslips. This enabled three separate treatments to be performed per adhesion run, and allowed for at least one control treatment to be included in each run.

Supplemented calf serum (Gibco) was heat-deactivated (56°C for 1 hour) prior to use in adhesion assays. The following lipoprotein fractions and apolipoproteins (purified from human plasma) were also tested for their ability to inhibit the adhesion of cockroach hemocytes to glass coverslips *in vitro*: VLDL (Sigma), LDL (Sigma, Calbiochem), HDL (Sigma), apoA-I (Sigma, Calbiochem), apoA-II (Calbiochem), apoB-100 (Fitzgerald), apoC-I (Calbiochem), apoC-II (Calbiochem), and apoE (Calbiochem). Purified (>99%) mouse IgG was purchased from Zymed.

Synthetic peptides of 15 to 43 amino acids in length, corresponding to sequences derived from the cDNA sequence of human apoB-100 (Yang *et al.*, 1986), were generously supplied by Dr. James Sparrow (University of Texas, Houston), with the

exception of peptide 3358-3372 which was purchased from Sigma). Their production has been described elsewhere (Chen *et al.*, 1988). The peptide sequences were 68-107, 228-269, 889-925, 3129-3171, 3358-3372, 3947-3983, 4154-4189, and 4507-4536.

ApoB-89 was a gift from Dr. Elaine Krul (Washington University, St Louis). The procedure she used to isolate apoB-89 LDL from an individual heterozygous for apoB-89/apoB-40 was a modification of the method used by Lee and Downs (1982). Plasma (containing proteolytic inhibitors) was adjusted to a final density of 1.04 g/ml with saline (150 mM NaCl, 1 mM EDTA). A density gradient was formed in a 40 ml QuickSeal tube (Beckman) as follows: 6 ml of saline with a density of 1.006 g/ml (density adjusted with KBr) was loaded into a tube and underlayered sequentially with 10 ml of saline (d=1.020 g/ml), 14 ml of plasma (d=1.040 g/ml) and finally 10 ml of saline (d=1.21 g/ml). Tubes were centrifuged in a Beckman 50.2 Ti rotor at 45 K for 24 hours at 12°C. After centrifugation, the gradient was pumped out of the tube from the bottom using a d=1.3 g/ml solution at a rate of 1 ml/min. The eluent was monitored for density and a fraction corresponding to $1.03 < d < 1.05$ g/ml was pooled (apoB-89 LDL). The LDL was washed once by adjusting the solution density to 1.02 g/ml and pelleting the LDL (45 K for 18 hours at 12°C). The LDL was then dialyzed against saline (pH 8.0).

3.3 Results

Both in the absence or presence of Ca^{2+}, calf serum almost completely inhibited the adhesion of cockroach hemocytes *in vitro* when added to experimental salines at a concentration of 1% (Fig. 3.1). Since calf serum contains large quantities of lipoproteins, and since lipophorin inhibited hemocyte adhesion, it was conceivable that inhibition of hemocyte adhesion was a general property of lipoproteins. Consequently, three major classes of lipoprotein purified from human plasma were tested for their ability to inhibit hemocyte adhesion (Fig. 3.2). Both LDL and VLDL strongly inhibited hemocyte adhesion, however a higher concentration of VLDL was required to achieve maximum inhibition. The finding that HDL did not significantly inhibit hemocyte adhesion indicated that inhibition of adhesion was not a property of all lipoproteins. In addition, 88%±7% of hemocytes adhered in 50 μg IgG/ml Sal I, indicating that this abundant plasma protein does not inhibit hemocyte adhesion (not shown).

These results, in conjunction with the knowledge that apoB-100 makes up approximately 90%, 54%, and 2% of the apolipoprotein content of LDL, VLDL, and HDL, respectively, suggested that apoB-100 was responsible for inhibiting adhesion. In order to test this, purified apolipoproteins were assayed for their ability to inhibit hemocyte adhesion (Fig. 3.3). Since delipidated apoB-100, like lipophorin, is

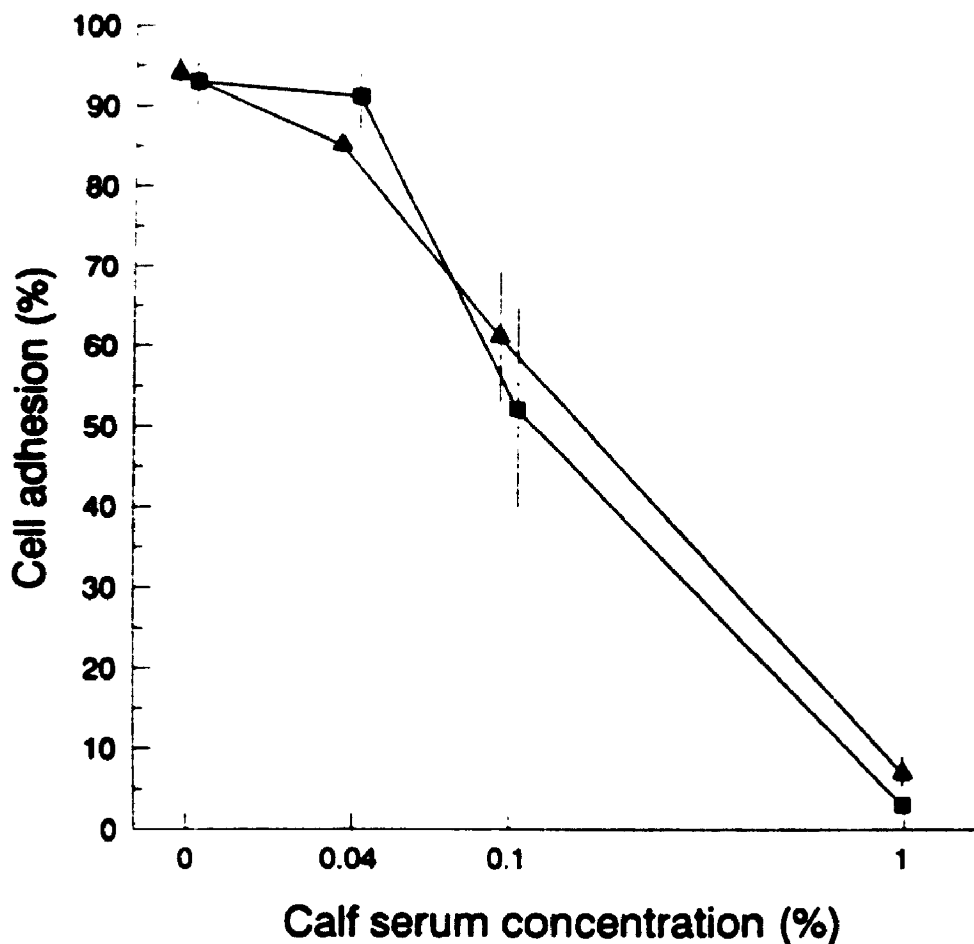


Figure 3.1 Concentration-dependence of calf serum on the inhibition of cockroach hemocyte adhesion *in vitro*. Almost no hemocytes adhered in 1% calf serum in either Sal I (absence of Ca^{2+} (squares)) or Perisal (presence of 2 mM Ca^{2+} (triangles)). Data points represent the mean ±SEM for 3 or more separate experiments.

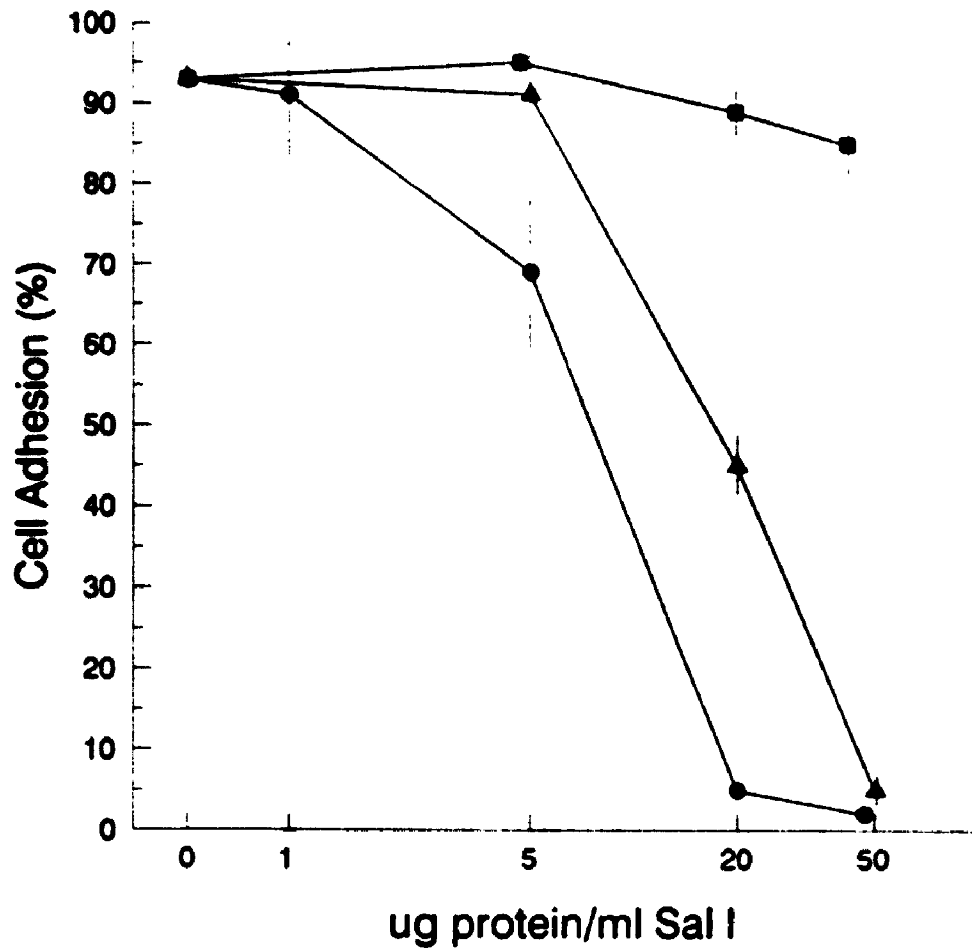


Figure 3.2 Concentration-dependence of human plasma lipoproteins on the inhibition of hemocyte adhesion. Both LDL (circles) and VLDL (triangles) inhibited hemocyte adhesion, but LDL was effective at a lower protein concentration. HDL (squares) did not significantly inhibit hemocyte adhesion even at 50 µg/ml. Data points represent the mean ±SEM for 3 or more separate experiments.

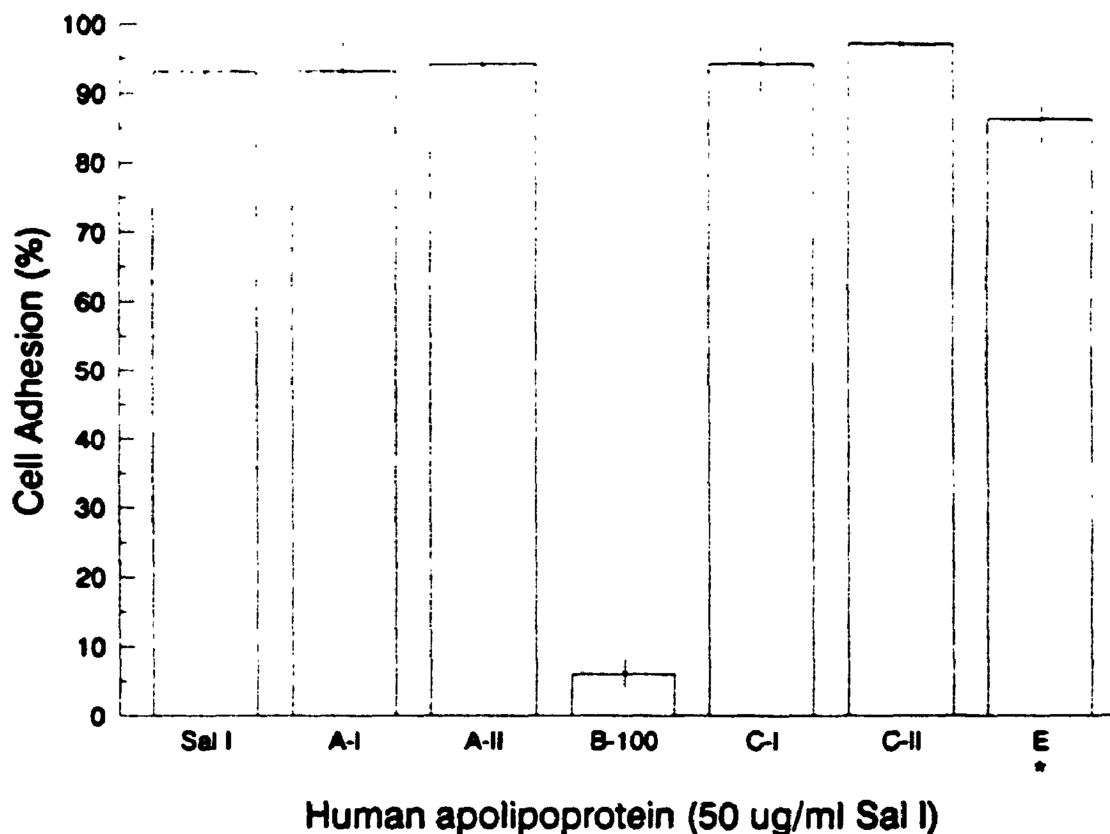


Figure 3.3 ApoB-100 inhibited hemocyte adhesion. Of the human apolipoproteins tested, only apoB-100 inhibited hemocyte adhesion at 50 μg/ml (* 20 μg/ml). All apolipoproteins tested had been delipidated prior to use, except for apoB-100, which is insoluble in aqueous solution once delipidated. Bars represent the mean ±SEM for 3 or more separate experiments.

insoluble in aqueous solution, highly purified LDL (Fitzgerald), with an apolipoprotein content of >99% apoB-100, was used. ApoB-100 (i.e. purified LDL), but not apoA-I, apoA-II, apoC-I, apoC-II, or apoE, strongly inhibited hemocyte adhesion.

The effect of apoB-100 concentration on hemocyte adhesion, both in the absence and presence of Ca^{2+}, was examined (Fig. 3.4). Whereas almost complete inhibition of adhesion by apoB-100 was achieved at a protein concentration of 20 µg/ml and 50 µg/ml in the absence of Ca^{2+}, 30%-40% of hemocytes adhered at the same protein concentrations when Ca^{2+} was present. This could be due to impurities in the apoB-100 sample which activate hemocytes in the presence of Ca^{2+}.

Eight synthetic peptides (15 to 43 amino acids in length), with sequences corresponding to different regions of apoB-100, were assayed in an attempt to map the site(s) on apoB-100 involved in inhibiting hemocyte adhesion. The total of 280 amino acids in these 8 peptides represents only 6% of the total number of 4536 amino acids in apoB-100. It was therefore fortunate to find that one of the these peptides (4154-4189), but none of the others, strongly inhibited hemocyte adhesion (Fig. 3.5). The amino acid sequence of this peptide includes an amphipathic α-helical region thought to participate in lipid-binding (Wei *et al.*, 1985) and is shown in Fig. 3.6. Although 4154-4189 inhibited hemocyte adhesion at approximately the same protein concentration as apoB-100 (Fig. 3.7), in molar terms apoB-100 was approximately 125x as

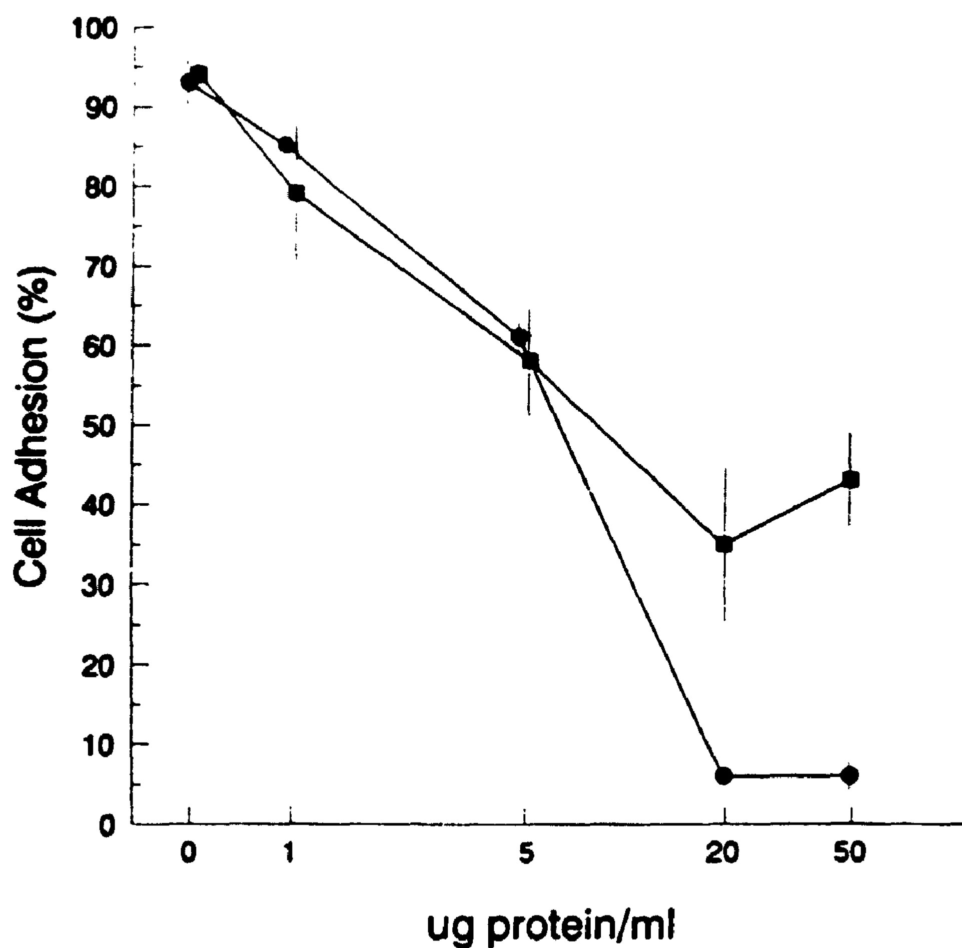


Figure 3.4 Concentration-dependence of apoB-100 on the inhibition of hemocyte adhesion. Whereas at the higher concentrations of apoB-100 <10% of hemocytes were adherent in Sal I (absence of Ca^{2+})(circles), >30% of hemocytes adhered in Perisal (2 mM Ca^{2+})(squares). Data points represent the mean ±SEM for 3 or more separate experiments.

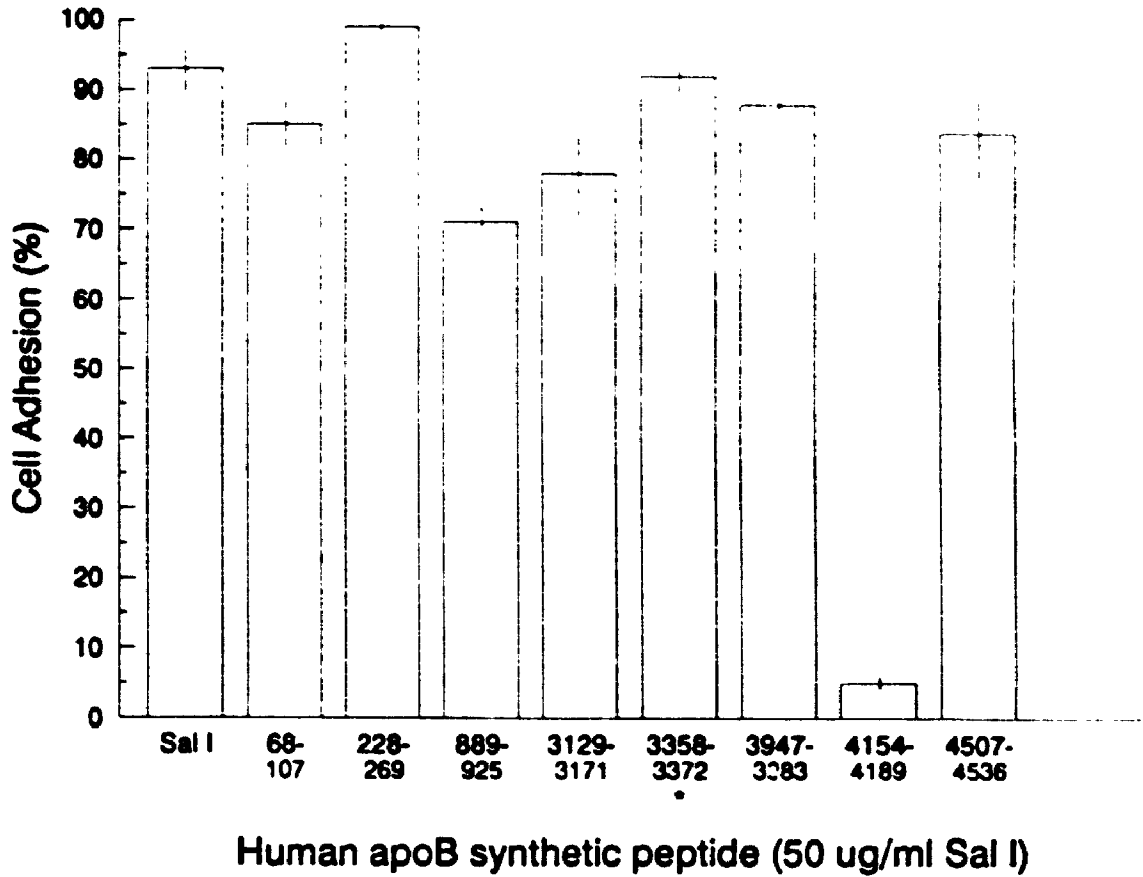


Figure 3.5 Human apoB-100 synthetic peptide 4154-4189 inhibited hemocyte adhesion. Of eight synthetic peptides corresponding to regions on apoB-100 tested at 50 μg/ml (* 100 μg/ml), only 4154-4189 strongly inhibited adhesion. Bars represent the mean ±SEM for 3 or more separate experiments.

4154 4160 4170 4180 4189

Glu Phe His Met Lys Val Lys His Leu Ile Asp Ser Leu Ile Asp Phe Leu Asn Phe Pro Arg Phe Gln Phe Pro Gly Lys Pro Gly Ile Tyr Thr Arg Glu Glu Leu

4165-4189

4173-4189

Figure 3.6 Amino acid sequence of the human apoB-100 synthetic peptide, 4154-4189. An amphipathic α-helical region (4154-4171) thought to be involved in lipid binding is underlined (from Wei *et al.*, 1985). Two other available synthetic peptides with shorter sequences (produced by James Sparrow) are also indicated (dotted lines).

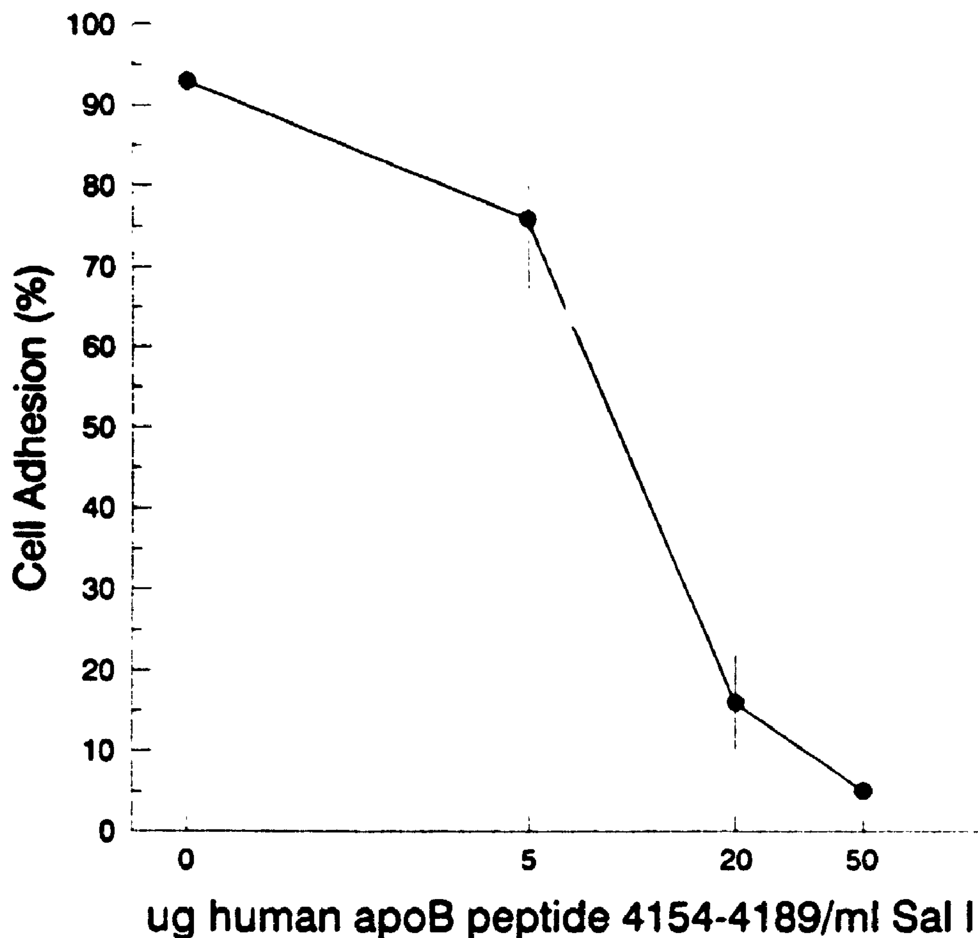


Figure 3.7 Concentration-dependence of apoB-100 peptide 4154-4189 on the inhibition of hemocyte adhesion. Although the peptide inhibits adhesion at approximately the same protein concentration, apoB-100 is 125x more effective than 4154-4189 in molar terms. Data points represent the mean ±SEM for 3 or more separate experiments.

effective as the peptide.

Individuals have been identified who produce truncated apoB-100s which lack part of the molecule at the C-terminus. One such truncated species, apoB-89, was of particular interest. It contains most of the apoB-100 sequence (4039 amino acids), but lacks 4154-4189. The finding that apoB-89 was inhibitory (Fig. 3.8) indicates that, in addition to 4154-4189, one or more sites exist on apoB-89 which are active in inhibiting hemocyte adhesion.

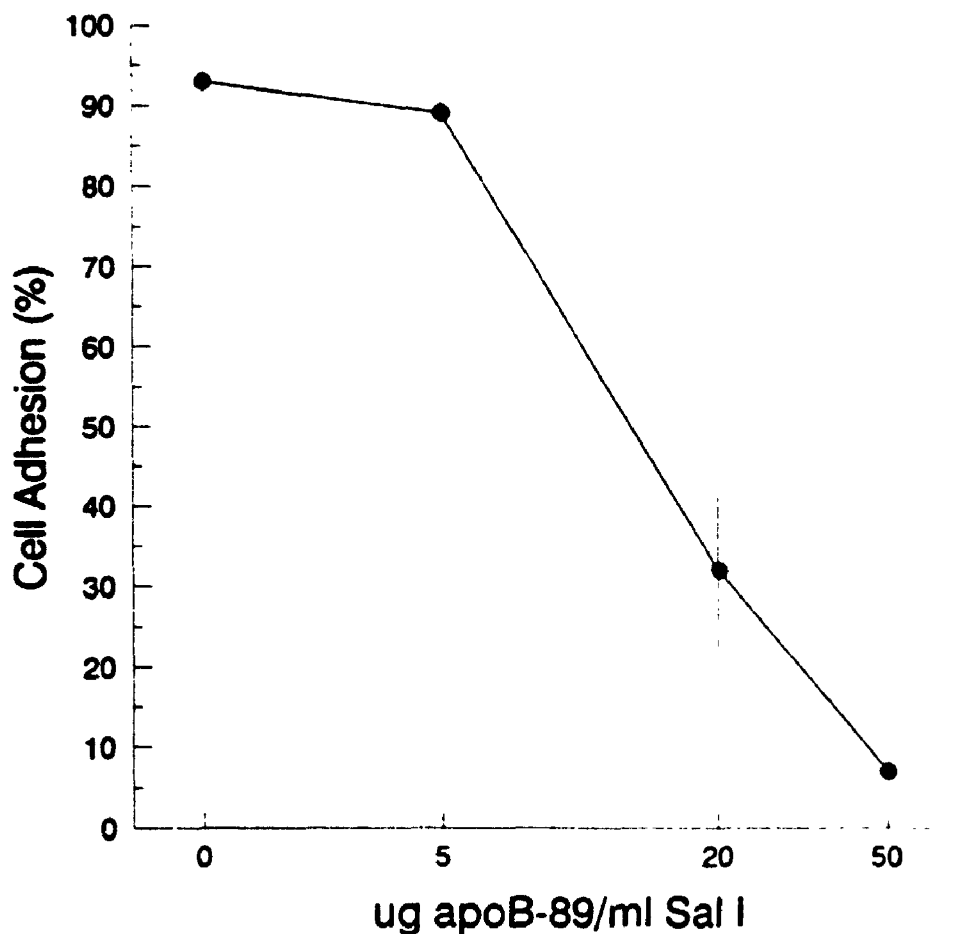


Figure 3.8 ApoB-89 inhibited hemocyte adhesion. ApoB-89, a truncated form of apoB-100 produced in certain individuals, does not contain the C-terminal 11% of the molecule. Since apoB-89 lacks the region of apoB-100 shown to inhibit adhesion (4154-4189), yet still inhibits hemocyte adhesion, one or more additional active sites must reside within apoB-89. Data points represent the mean ±SEM for 3 or more separate experiments.

3.4 Discussion

The discovery that calf serum inhibited the adhesion of cockroach hemocytes *in vitro*, as did lipophorin from insect plasma, suggested that this was perhaps a general property of lipoproteins. In order to address this issue, I narrowed my focus to examine the effects of different lipoprotein classes (i.e. VLDL, LDL, and HDL). It remains to be determined whether other serum components (i.e. non-lipoproteins) are also capable of inhibiting hemocyte adhesion.

ApoB-100 from human plasma, but no other human apolipoprotein tested, was found to inhibit the adhesion of cockroach hemocytes *in vitro*. ApoB has been intensively studied due to a strong correlation in humans between a high concentration of apoB-100 in plasma and the development of atherosclerosis (Brunzell *et al.*, 1984). Whereas the complete lipophorin amino acid sequence has yet to be determined, the sequences of many vertebrate lipoproteins, including human apoB-100 (e.g. Yang *et al.*, 1986), have been published. By utilizing synthetic apoB-100 peptides developed in other laboratories (primarily to pinpoint regions on apoB-100 involved in its binding to the LDL-receptor), I have been able to locate regions on apoB-100 involved in inhibiting hemocyte adhesion. Since there is considerable evidence suggesting that apoB-100 and lipophorin are derived from a common ancestor (discussed below), information obtained for apoB-100 may provide clues as to the location of the site(s) on lipophorin

responsible for inhibiting hemocyte adhesion. These findings also hint at a role for apoB-100 in vertebrates in addition to that of lipid transport and regulation.

3.4.1 Identification of regions on apoB-100 involved in inhibiting hemocyte adhesion

3.4.1a Synthetic apoB-100 peptides

Eight synthetic peptides (15-43 amino acids in length) whose sequences were derived from the cDNA sequence of human apoB-100 were tested for their ability to inhibit hemocyte adhesion. One of these, 4154-4189 (36 amino acids), strongly inhibited adhesion. Since neither lipophorin nor apoB-100 are soluble in aqueous solution once delipidated, it was not possible to determine whether lipid associated with these molecules was involved in inhibiting hemocyte adhesion. However, the finding that an apoB-100 synthetic peptide inhibited adhesion suggests that lipid on apoB-100, and perhaps on lipophorin as well, is not required for this activity. It should be kept in mind, though, that apoB-100 peptide 4154-4189 contains an amphipathic α-helical region from amino acid residue 4154 to 4171 (Fig. 3.6)(Wei *et al.*, 1985). Since amphipathic α-helices are involved in lipid-binding (Segrest *et al.*, 1974), inhibition of hemocyte adhesion might involve lipid-binding by this part of the peptide, possibly to lipids found on the plasma membrane. The

fact that amphipathic α-helices are found in one of the other apoB peptides (4507-4536)(Wei *et al.*, 1985) and in each of the other apolipoproteins tested (apoA-I, apoA-II, apoC-I, apoC-II, and apoE)(Segrest *et al.*, 1992), none of which inhibited hemocyte adhesion, however, does not support the notion that lipid-binding *per se* has a role in the inhibition of hemocyte adhesion. If the apoB-100 peptide 4173-4189 (Fig. 3.6), which lacks the amphipathic α-helix found in 4154-4189, can be shown to inhibit adhesion, this will provide conclusive proof that lipid-binding is not involved.

ApoB-100 and the 4154-4189 peptide showed approximately equal concentration dependence when measured in terms of μg protein/ml required to inhibit hemocyte adhesion. In molar terms, however, apoB-100 was approximately 125x as effective at inhibiting hemocyte adhesion as compared with the peptide. One possible explanation for this discrepancy is that inhibition of adhesion depends upon a particular conformation found in apoB-100 which is partially disrupted in 4154-4189, thus resulting in a decreased efficiency of the peptide. A second interpretation is that inhibition of hemocyte adhesion by apoB-100 and 4154-4189 does not involve a specific interaction with hemocytes (e.g. with a plasma membrane receptor), but rather the non-specific coating of the glass substrate.

3.4.1b apoB-89

Under normal circumstances, human apoB is secreted in two

forms. One of these, apoB-100 (4536 amino acids), is produced in the liver, and secreted into the bloodstream with associated lipid as VLDL. There it is converted to LDL and removed from circulation via LDL receptor-mediated endocytosis. The other form, apoB-48 (2152 amino acids), is identical to the N-terminal 48% of apoB-100, and is secreted from the small intestine in association with chylomicrons (Chan, 1992). In addition to apoB-100 and apoB-48, certain individuals produce truncated species of apoB. One of these truncated forms, apoB-89 (4039 amino acids), was of particular interest to me since it lacks the C-terminal 11% of apoB-100 (Krul *et al.*, 1989; Talmud *et al.*, 1989), and, therefore, contains most of apoB-100 but not the stretch of amino acids from 4154 to 4189 which inhibited hemocyte adhesion. If 4154-4189 represents a unique site on apoB-100 capable of inhibiting hemocyte adhesion, then it would be expected that apoB-89 would not inhibit adhesion. The finding, however, that apoB-89 did inhibit adhesion indicates that at least one other site, other than 4154-4189, exists which is also capable of inhibiting hemocyte adhesion.

3.4.1c <u>Determining the site(s) on apoB-89 involved in inhibiting hemocyte adhesion</u>

By testing apoB-48 and truncated forms of apoB other than apoB-89 (Krul *et al.*, 1992) it might be possible to obtain an estimate of where the other active site(s) within apoB-89 are found. However, because apoB-100 is such a large protein (4536

amino acids), pinpointing the exact location of these site(s) is a difficult task. The presence of several regions within apoB-100 whose amino acid sequences are similar to 4154-4189, however, might provide a clue as to where other region(s) responsible for inhibiting hemocyte adhesion are located. The identification of a large number of long (>70 residues) and short internal repeats within apoB-100 suggests that its primary sequence was derived largely from internal duplication (Yang *et al.*, 1986; De Loof *et al.*, 1987). The region from 2262-2297, part of a larger repeated region, is similar to 4154-4189 (Yang *et al.*, 1986). In addition, there exist eight amphipathic α-helical regions within apoB-100 which are similar in amino acid sequence to the one found in the $-NH_2$ portion of 4154-4189 (i.e. from residue 4154 to 4171). One or more of these regions similar in sequence to 4154-4189 might be involved in inhibiting hemocyte adhesion. None of the apoB-100 peptides tested correspond to any of these regions. Synthetic apoB-100 peptides that do overlap several of the above-mentioned regions of the apoB-100 molecule have been produced by others (Innerarity *et al.*, 1987) and could be tested.

3.4.1d Monoclonal antibodies raised against apoB-100

Monoclonal antibodies have been used to study the general structure of apoB (Chatterton *et al.*, 1991), and more specifically to localize the low density receptor-binding domain on its surface (Milne *et al.*, 1989; Pearse *et al.*,

1990). There are therefore a large number of monoclonal antibodies to apoB-100 available whose binding sites have been well-defined. These antibodies might be useful in identifying regions on apoB-100 involved in inhibiting hemocyte adhesion.

3.4.2 Do apoB-100 and lipophorin inhibit hemocyte adhesion by the same mechanism?

Similar solubility properties, secondary structure and amino acid sequence of lipophorin and apoB-100 suggest that the two proteins may have a common origin. Insect lipophorin (Shapiro *et al.*, 1984b) and vertebrate apoB-100 (Cardin *et al.*, 1982) are both insoluble in aqueous buffers following delipidation. In addition, both proteins appear to contain large amounts of β-pleated sheet as part of their secondary structure (Kashiwazaki and Ikai, 1985; Chan, 1992). The lipophorin gene from two species of insect, *Locusta migratoria* (by Dick van der Horst, Utrecht, The Netherlands) and *Manduca sexta* (by Michael Wells, Tucson, Arizona), have been partially sequenced. Available sequence data from both insects indicate that lipophorin is more similar to apoB than to any other protein (van der Horst *et al.*, 1993; Michael Wells, personal communication).

The two models proposed to explain the way in which lipophorin inhibits hemocyte adhesion *in vitro* also apply to apoB-100 (see Chapter 2, Fig. 2.17). ApoB-100 could inhibit

adhesion by binding to the hemocyte surface (perhaps to a receptor in the plasma membrane), or alternatively by coating the glass substrate. Evidence supporting a common origin for lipophorin and apoB-100, coupled with the possibility that 4154-4189 (and one or more other as yet unidentified regions of apoB-100) inhibit hemocyte adhesion by binding to a receptor on the hemocyte plasma membrane, suggest that the possible conservation of these sequences between insects and vertebrates is due to functional constraints. While this notion is speculative at present, it does raise an intriguing question: does the 4154-4189 sequence on apoB-100 have a function in humans related to its ability to inhibit cell-substrate adhesion of insect blood cells? Platelets have been shown to adhere *in vitro* to lipoproteins, including LDL (Kowalska *et al.*, 1990), and preliminary results indicate that LDL does not inhibit cell-substrate adhesion of isolated mammalian neutrophils *in vitro* (personal observation). It would be worthwhile to extend these observations by examining the effect of LDL on the adhesion of other leukocyte types (e.g. lymphocytes), especially in light of the finding that LDL suppresses mitogen-induced lymphocyte activation (Hui *et al.*, 1980).

Chapter 4: Rapid Formation of GJs Between Hemocytes *In Vitro*

4.1 Introduction

Insect hemocytes normally circulate freely in the hemocoel. During the cellular immune response against parasit or other large foreign objects in the hemocoel, hemocytes encapsulate invaders by adhering and flattening onto them and onto one another, forming a capsule many cell layers thick (Grimstone et al., 1967; Gotz, 1986). Hemocytes form various intercellular junctions during encapsulation, including gap junctions (GJs) (Baerwald, 1975; Norton and Vinson, 1977; Han and Gupta, 1989) which are plasma membrane structures composed of a lattice of cell-to-cell channels that directly connect the cytoplasms of cells within most metazoan tissues. Individual channels form when hemi-channels, or connexons, from each of two cells line up end to end to form a hydrophilic conduit. GJs are considered to be involved in electrical and metabolic coupling, and direct intercellular signalling (Loewenstein, 1981; Bennett et al., 1991).

In *Periplaneta americana,* GJa1 plaques were present on freeze-fracture replicas of hemocyte capsules that formed around synthetic implants 48 hours post-implantation (Baerwald, 1975). Hemocytes comprising a 72-hour-old capsule were shown to be strongly dye- and electrically-coupled (Caveney and Berdan, 1982). The possibility that GJs form between hemocytes *in vitro* would offer a novel model system for studying GJ formation. In most other studies, cells with

fully-formed GJs were dispersed and then re-aggregated to allow GJs to re-form (Preus et al., 1981; Chow and Young, 1987). Hemocytes, however, form GJs *de novo* since these normally monodispersed cells presumably had not formed GJs previously.

In this chapter I present both structural and functional evidence showing that hemocytes form GJs rapidly, almost instantaneously, after bleeding *in vitro*. E-face GJal plaques were visible in freeze-fractured hemocyte aggregates 5 min after bleeding. A carboxyfluorescein diacetate (CFDA) dye-transfer assay was developed to demonstrate that functional coupling could be detected between newly contacting hemocytes within 3 min of bleeding. Even hemocytes derived from distantly related insects (cockroach and moth) rapidly form functional GJs with one another. With this CFDA assay I was able to examine quantitatively the effects of trypsinizing hemocytes on the rate at which dye-coupling occurred. By slowing down hemocyte activation *in vitro* with lipophorin, Dennis Churchill and I used the double whole-cell patch-clamp technique to study the properties of single GJal channels during their formation. Within 1 second of manipulating hemocytes into contact clear step-like transitions in the junctional current were measurable, reflecting the accretion of single channels to the growing GJ.

4.2 Materials and methods

4.2.1 Insects

Periplaneta americana was reared as described in section 2.2.1, and *Calpodes ethlius* as previously described (Locke, 1970). Other insects were kept at 25°C and fed live cockroaches and mealworms (*Mantis religiosa*), lettuce (*Gryllus pennsylvaticus*), or ground Dog Chow mixed with refined sugar (*Leucophaea maderae*). All insects were supplied with water *ad libitum*.

4.2.2 Freeze-Fracture

Cockroaches (*Periplaneta americana*) were bled through cut antennae into Carlson's saline (Mitsuhashi, 1982: 120 mM NaCl, 44.4 mM glucose, 2.7 mM KCl, 1.8 mM $CaCl_2$-$2H_2O$, 1.7 mM NaH_2PO_4-$2H_2O$, 1.1 mM $MgCl_2$-$6H_2O$, 0.6 mM $NaHCO_3$, pH to 7.2 with NaOH). Hemocytes immediately formed large clumps which were easily manipulated. After the indicated time (0-180 min), cells were fixed in 2.5% glutaraldehyde/0.1 M sodium cacodylate (2x30 min), rinsed in 0.1 M sodium cacodylate (x2), and soaked in 30% glycerol/0.1 M sodium cacodylate (2x30 min). Hemocytes were then frozen in liquid nitrogen-cooled Freon-22. Platinum-carbon replicas were made in a Balzers BAF 301 Freeze-Etch Unit (Balzers, Liechtenstein) according to the procedure of Shivers and Brightman (1976). Replicas were examined in a Philips 201 electron microscope operating at an accelerating voltage of 60 kV.

4.2.3 CFDA assay

Cockroaches were punctured at the base of a mesothoracic leg and 1 μl of hemolymph quickly collected into a glass micropipet containing 5 μl of Sal I. This was immediately added to 500 μl of Sal I, spun at 372xg for 30 sec, the pellet of cells resuspended in 500 μl of Perisal containing 25 μg CFDA (Sigma)(added from a 50 mg CFDA/ml anhydrous DMSO stock solution), and pipetted onto a coverslip dish (consisting of a teflon O-ring clamped onto a glass coverslip by a metal plate). Excess CFDA and non-adherent hemocytes were rinsed away after 10 min with Perisal (12x500 μl rinses) for Ca^{2+}-containing experiments, or with Sal I (12x500 μl rinses) for Ca^{2+}-free experiments. From a second cockroach, 10 μl of hemolymph was then collected into a micropipet containing 10 μl of Sal I, cells pelleted as before, and resuspended in 500 μl of Perisal or Sal I used as the final (i.e. twelfth) rinse. Dye-transfer from fluorescently-labelled to unlabelled cells was monitored every 3 min for 30 min using a Zeiss Axiovert 35 Inverted Phase Microscope with standard carboxyfluorescein fluorescence optics (Carl Zeiss Canada, Don Mills, Ontario).

In CFDA experiments in which interspecific coupling was examined, *Periplaneta* hemocytes were labelled, and unlabelled hemocytes from another insect species then added. Hemocytes were obtained from the following insects: *Leucophaea maderae* (Dictyoptera), *Gryllus pennsylvaticus* (Orthoptera), *Mantis religiosa* (Mantodea), and fifth instar larvae of *Calpodes ethlius* (Lepidoptera). For experiments involving *Calpodes*,

Calsal (75 mM glucose, 25.5 mM KCl, 20.7 mM $MgCl_2$-$6H_2O$, 10.3 mM NaCl, 10 mM HEPES, 2.7 mM $CaCl_2$-$2H_2O$, pH to 6.8 with NaOH) was used in place of Perisal.

4.2.4 Quantitative CFDA Assay

The rate of formation of functional GJ channels between hemocytes was quantified by measuring the rate of loss of fluorescence from labelled hemocytes to unlabelled contacting neighbours. Whereas fluorescence is lost slowly and steadily from cells not coupled to other cells, the drop in fluorescence intensity from labelled hemocytes occurs much more rapidly when they form GJs with, and pass dye to, unlabelled hemocytes. Hemocytes were labelled as described above and for 30 min following the addition of unlabelled cells the fluorescence image of a single field of view was videotaped (Sony TVO-9000 3/4" VCR) for 2 seconds at 2 minute intervals using an MTI SIT 66 television camera attached to the cineport of a Zeiss Axiovert 35 Inverted Microscope with epifluorescence optics. During tape playback, the fluorescence intensity for each labelled cell was measured (Safranyos and Caveney, 1985) at 2 minute intervals. The fluorescence intensity for each labelled cell was normalized by designating the absolute fluorescence intensity after the first interval (i.e. 2 minutes after the addition of unlabelled cells) as 100% for that cell. The fluorescence intensity (expressed as a percentage) for the 3 to 9 labelled cells within a field of view was averaged for each time point. Each experiment was

performed five or more times and the mean and standard error of each data set determined.

4.2.5 Double whole-cell voltage clamp

Within several minutes of settling out of solution, *Periplaneta americana* hemocytes adhere and flatten extensively onto glass coverslips *in vitro*, either in the presence or absence of Ca^{2+} (see Chapter 2). Initial attempts by Dennis Churchill in our laboratory to patch-clamp these highly flattened cells proved unsuccessful due to the extreme difficulty of obtaining high-resistance seals and break-ins. The subsequent discovery that lipophorin inhibits the adhesion and flattening of hemocytes *in vitro* (Chapter 2), however, made it possible to perform patch-clamp experiments on these cells.

In order to reduce hemocyte flattening, hemocytes were bled and incubated in saline containing 5 μg lipophorin/ml. Although the cells had a more rounded morphology when incubated in this concentration of lipophorin, it was still difficult to obtain high-resistance seals, possibly due to lipophorin adhering to the patch-pipets. However, by coating coverslips for 30 min with 50 μg lipophorin/ml, hemocyte flattening was delayed sufficiently so that high-resistance seals could be obtained. In order to study GJ formation, single cells were whole-cell voltage-clamped and manipulated into contact. For experimental details see Churchill *et al.* (1993).

4.3 Results

4.3.1 Freeze-fracture

Aggregated, freshly-bled, *P. americana* hemocytes were incubated for between 0 and 180 minutes in saline *in vitro*, fixed, and then freeze-fractured to determine if GJs would form *in vitro*, and if so, how quickly. Typical P-face and E-face views of the plasma membrane of hemocytes bled directly into fixative are shown in Fig. 4.1. As in other cell types examined by freeze fracture, and as previously demonstrated for *P. americana* hemocytes (Baerwald,1974), the P-face of the plasma membrane contains a much higher concentration of intramembranous particles than does the E-face. Baerwald (1974) never observed clustering of P-face or E-face particles on the plasma membranes of hemocytes bled directly into fixative. While I also did not see any clusters of E-face particles on hemocytes treated in this way, I did sometimes observe strings of P-face particles (Fig. 4.2A, 4.2B). More numerous and longer P-face strings were seen on hemocytes incubated in saline prior to fixation (Fig. 4.2B). Discontinuous linear arrays of P-face particles have been observed in other insect tissues (Lane and Skaer, 1980).

Incipient GJal plaques, seen as loose aggregates of E-face particles, were visible on freeze-fracture replicas of hemocytes fixed 5 min post-bleeding (Fig. 4.3A). Larger GJal plaques with more-densely packed intramembranous particles formed within 10 min post-bleeding (Fig. 4.3B). Occasionally

Figure 4.1 P-face and E-face views of the plasma membrane of *Periplaneta* hemocytes. The density of intramembranous particles (i.e. per unit area) is much higher on the P-face (A) as compared with the E-face (B). This is advantageous since it makes the identification of gap junctional plaques on freeze-fracture replicas, which for insect cells partition with the E-face, easy on hemocytes. No clusters of E-face particles were observed on hemocytes bled directly into fixative. Bar=0.2 μm.

Figure 4.2 P-face strings of intramembranous particles on the hemocyte plasma membrane. (A) Linear alignments (strings) of P-face particles are found on the P-face of hemocytes bled directly into fixative. (B) Hemocytes incubated in saline prior to fixation exhibit longer and more numerous P-face strings. Bars=0.2 μm.

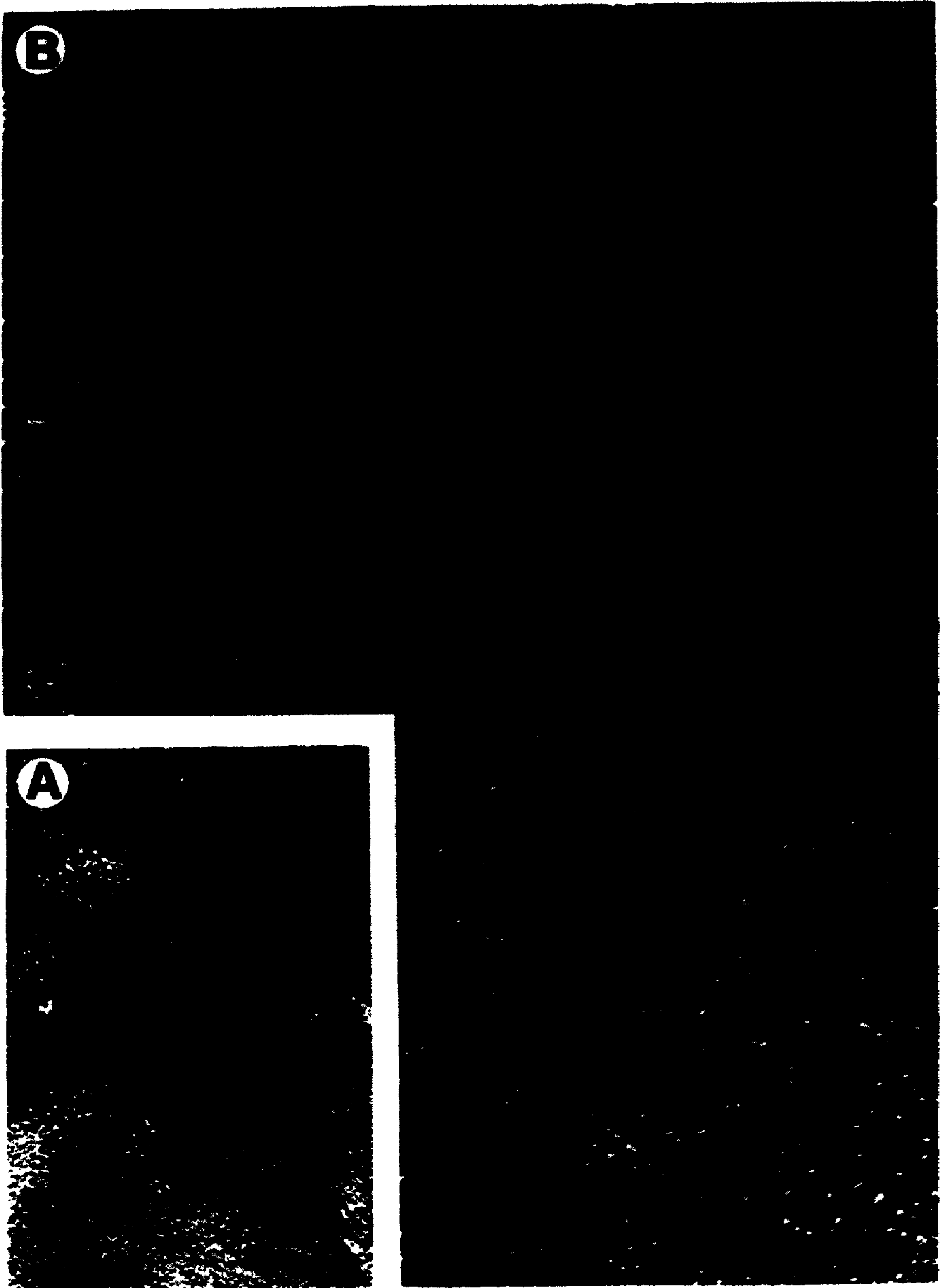
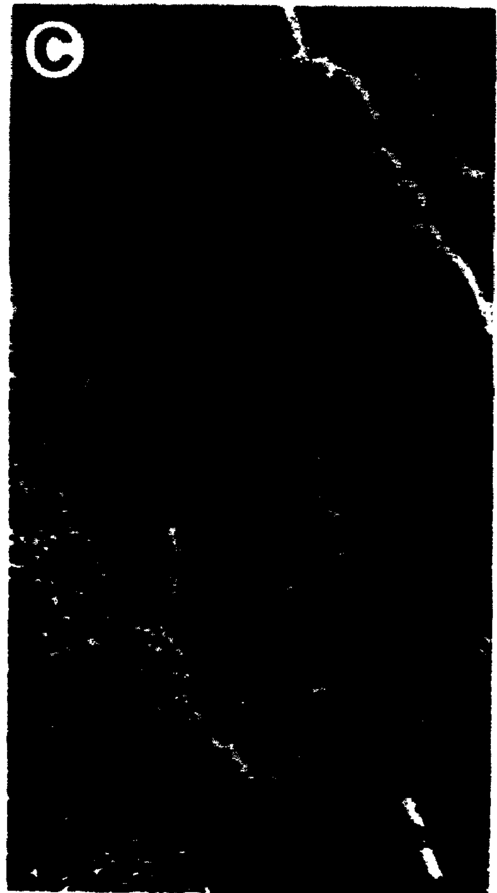

B
A

Figure 4.3 Formation of GJa1 plaques on the hemocyte plasma membrane. (A) Streams of E-face particles coalescing to form a GJa1 plaque can be seen on hemocytes fixed 5 minutes post-bleeding. (B) Plaques with more densely packed intramembranous particles are visible on hemocytes fixed 10 min post-bleeding. (C) This replica of hemocytes fixed 180 minutes post-bleeding shows the P-face pits (arrows) sometimes seen at the corresponding sites of E-face GJa1 plaques. Bar=0.2 μm.

A

B


C

clusters of P-face pits, corresponding to E-face plaques, were observed (Figure 4.3C).

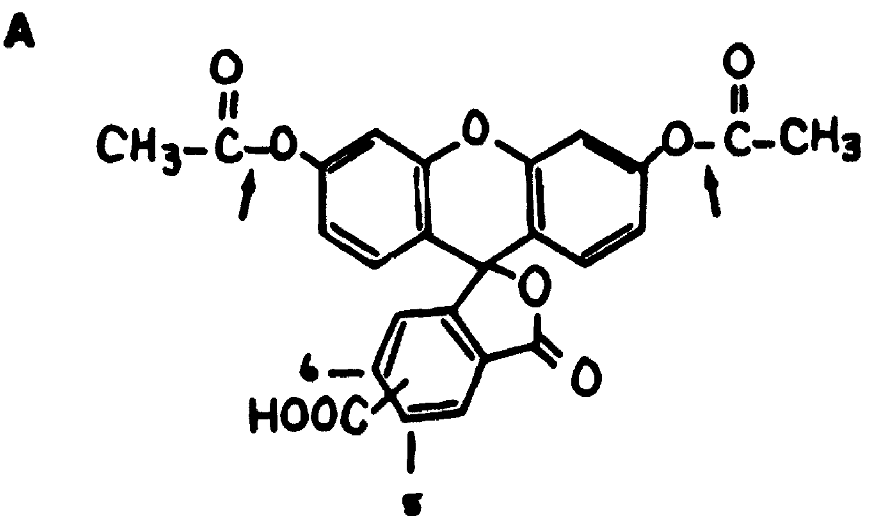
4.3.2 CFDA Assay

4.3.2a GJ formation between *P. americana* hemocytes

A dye-transfer assay demonstrated that hemocytes could form functional GJs *in vitro* (n>50 replicate experiments). Hemocytes were incubated in the nonfluorescent, non-polar reagent CFDA, which enters cells freely. Within the hemocytes CFDA was cleaved by esterases to form the hydrophilic fluorescent dye, carboxyfluorescein, which remained trapped within the cells (Figure 4.4). Unlabelled hemocytes bled from a 2nd insect were then added and as they settled onto the coverslip and made contact with labelled cells, dye-transfer from labelled to unlabelled cells was monitored. Typically, dye-transfer was apparent between many labelled/unlabelled cell combinations within 9 min of adding unlabelled cells (Fig. 4.5). Occasionally, dye-transfer was observed within 3 min. Often groups of 10 or more contacting unlabelled cells formed around a labelled cell. Dye passed to all the cells within such groups, including cells not directly in contact with the pre-loaded cell (i.e. second- and third-order neighbours). Small areas of contacting plasma membrane are sufficient for GJs to form since dye-transfer was observed between cells which were connected only by fine filopodia. On occasion, dye-transfer occured between cells which

Figure 4.4 Outline of the CFDA assay. (A) Structure of carboxyfluorescein diacetate (CFDA). CFDA readily enters cells where it is cleaved (arrows) by esterases to form the hydrophilic dye carboxyfluorescein, which is trapped within cells. (B) Outline of the the CFDA assay used for detecting the formation of GJal coupling.

A



B

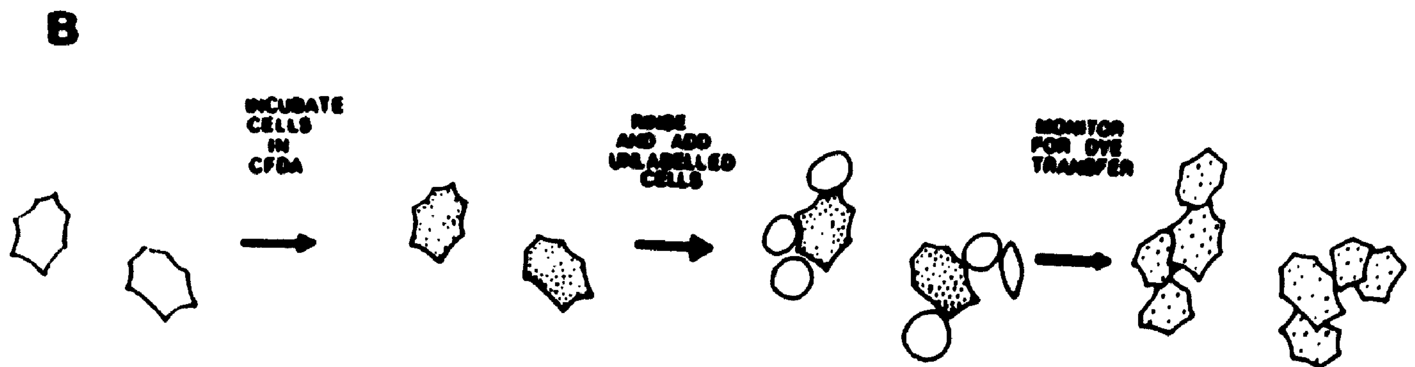
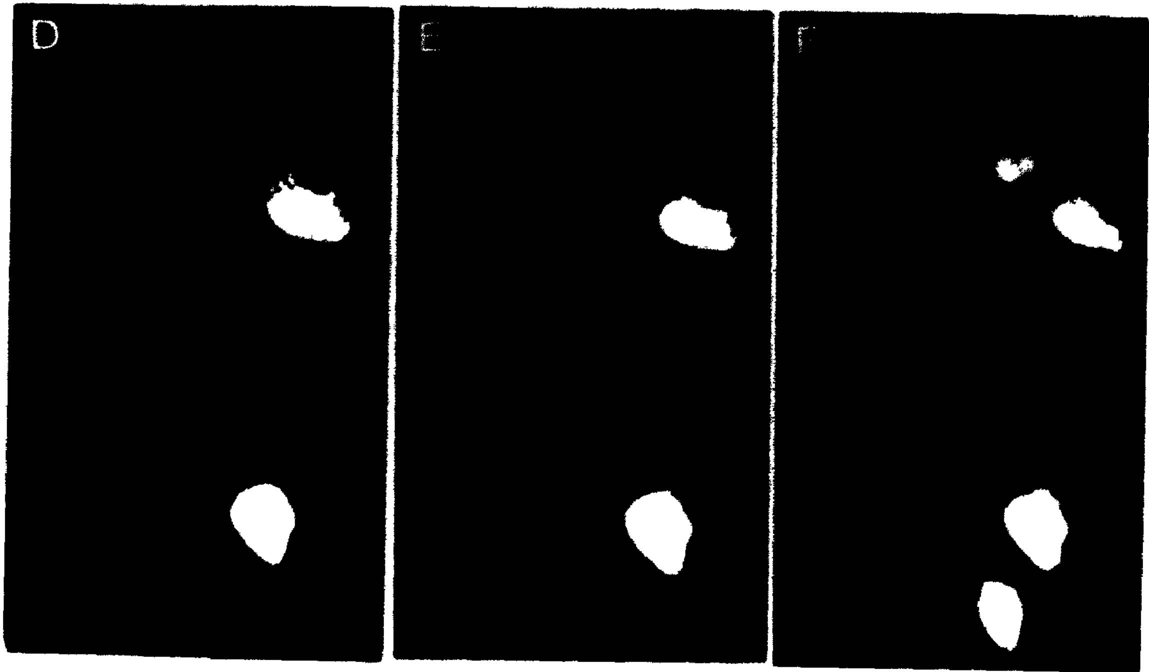


Figure 4.5 CFDA assay demonstrates the rapid *de novo* formation of GJs between *Periplaneta* hemocytes. Phase contrast (A,B,C) and fluorescence (D,E,F) micrographs were taken 6 min (A and D), 9 min (B and E), and 12 min (C and F) after the addition of unlabelled hemocytes to already-flattened, fluorescently-labelled hemocytes. Transfer of dye from labelled (arrows) to unlabelled (arrowheads) cells is visible within 9 min of adding unlabelled cells. Bar=10 μm.


D
E
F

subsequently migrated away from one another so that they were no longer in contact, indicating that hemocytes are capable of both forming and breaking GJs *in vitro*. In the absence of Ca^{2+} (i.e. in Sal I) dye-passage was also observed between labelled and unlabelled cells (n=3 replicate experiments), within 6 min.

4.3.2b Interorder GJal coupling between insect hemocytes

The CFDA assay was also used to demonstrate that *P. americana* hemocytes can form functional GJs with hemocytes derived from other insects *in vitro*. Dye-coupling was detected in under 15 min between labelled *P. americana* (order Dictyoptera) hemocytes and unlabelled hemocytes from *Calpodes ethlius* (Lepidoptera)(n=4 replicate experiments), *Leucophaea maderae* (Dictyoptera)(n=1), *Gryllus pennsylvaticus* (Orthoptera)(n=1), and *Mantis religiosa* (Mantodea)(n=1). *P. americana* is an excellent insect to use for the CFDA assay since it has a relatively high blood volume and cell density, and its hemocytes retain the dye when labelled with CFDA. Some insects were difficult or impossible to use for this assay since their hemocytes quickly lost the dye (*Leucophaea maderae*) or their hemocytes could not be obtained in sufficient numbers (*Drosophila melanogaster*).

4.3.2c Rate of dye-passage between contacting hemocytes

While the CFDA assay was useful for demonstrating that the GJs formed by hemocytes *in vitro* are functional, it was

clear that it would have greater application if it could be modified so as to measure intercellular coupling quantitatively. This simple, non-invasive technique could then be used to examine the effects of various treatments on GJ formation. I developed an assay to measure the rate of GJ formation by monitoring the rate of loss of fluorescence from CFDA-labelled hemocytes as they form GJs with and pass dye to unlabelled cells. The fluorescence intensity of labelled cells not participating in GJ formation (i.e. incubated in the absence of unlabelled cells) decreased by 50% in 20 min due to leakage of carboxyfluorescein from the cells (Fig. 4.6). The drop to 50% fluorescence intensity, however, occurred in only 8 min when labelled cells were incubated with an excess of unlabelled hemocytes (ratio ≈ 1:10). This is due to the passage of dye through newly-formed GJs to the unlabelled cells.

The effects of trypsinizing hemocytes on the establishment of GJα1 coupling were examined by this method. When both labelled and unlabelled hemocytes were treated with 0.1% trypsin, the rate of dye loss from the labelled hemocytes was dramatically reduced (50% loss in 20 min) as compared to non-trypsinized cells (50% loss in 6 min)(Fig. 4.7). Exposing only the fluorescently-labelled hemocytes to trypsin did not alter the rate of dye-transfer between the two groups of cells. Adding soybean trypsin inhibitor prior to the addition of the unlabelled hemocytes also allowed coupling to develop normally. Thus, inhibition of GJα1 coupling between hemocytes

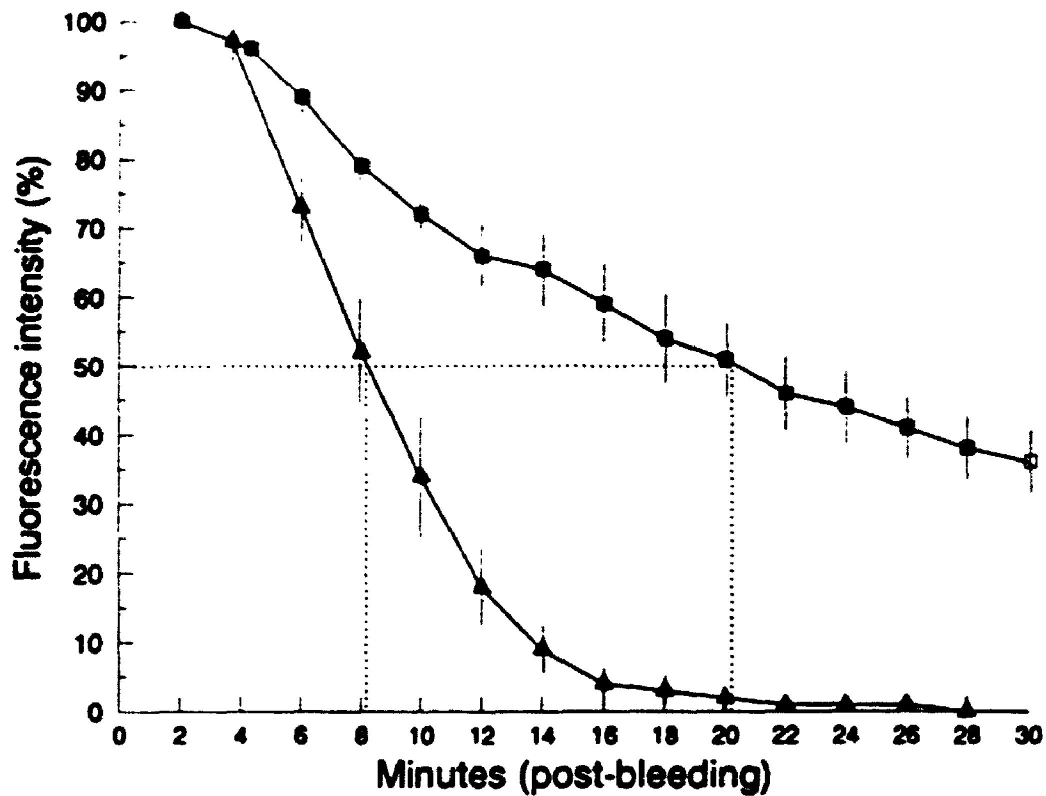


Figure 4.6 Decrease in fluorescence intensity in labelled cells can be used to measure the rate of formation of functional GJs. The loss of fluorescence from labelled hemocytes occurs more quickly when they are incubated with unlabelled hemocytes (50% loss in 8 min; closed triangles) than when they are incubated in the absence of unlabelled cells (50% loss in 20 min; closed squares) due to the passage of dye to unlabelled cells via gap junctions. Data points represent the mean ±SEM for 3 or more separate experiments.

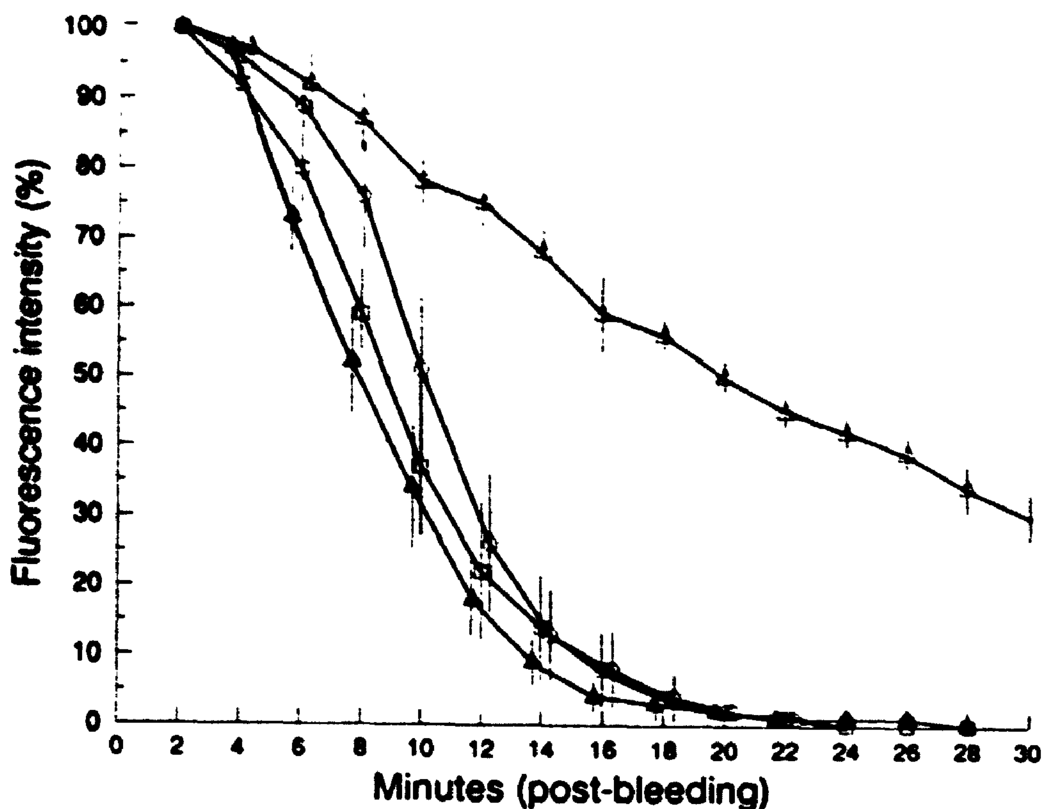


Figure 4.7 Dye-transfer via GJs is diminished if both labelled and unlabelled hemocytes are trypsinized. To examine the effects of trypsin on the establishment of GJal coupling, fluorescently labelled hemocytes were incubated for 10 min in saline containing 0.1% trypsin followed by the addition of unlabelled hemocytes (i.e. both populations of cells were exposed to trypsin)(open triangles). Trypsinization of labelled and unlabelled cells results in diminished coupling (i.e. a slower loss of fluorescence from labelled cells) as compared with cells not exposed to trypsin (closed triangles). If, however, only the labelled cells are exposed to active trypsin, either by rinsing trypsin away (open squares) or adding 0.1% soybean trypsin inhibitor (open circles) prior to adding unlabelled cells, the rate of formation of functional GJs is not significantly affected. Data points represent the mean ±SEM for 3 or more separate experiments.

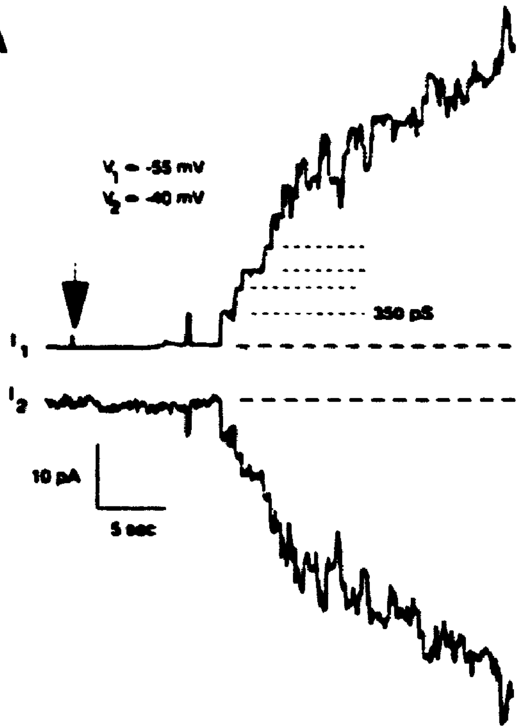
requires that both populations of cells be exposed to active trypsin.

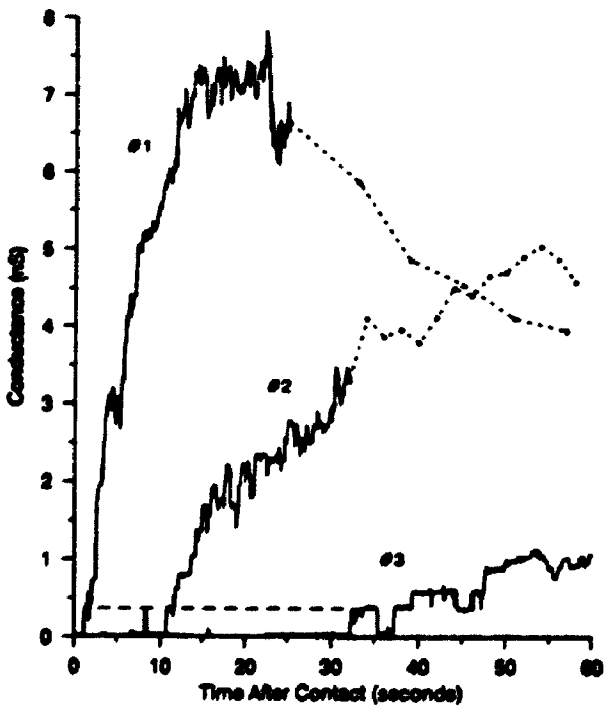
4.3.3 Double whole-cell voltage-clamp

After 30 minutes, some hemocytes retained a discoid morphology on lipophorin-coated coverslips, while most remained rounded and only loosely adherent (Fig. 4.8). Under these conditions it became possible to obtain patch-clamp recordings for up to 45 min and occasionally up to 1 to 2 hours after plating the cells. In order to study GJ formation, single cells were whole-cell voltage-clamped and manipulated into contact. Interestingly, non-adherent discoid cells failed to form GJs when pushed together, apparently indicating that 'resting' hemocytes must be activated in order to form GJs (discussed in section 4.4.4). The rounded hemocytes, however, when teased free with pipets from their loose attachments to lipophorin-coated glass coverslips and manipulated into contact, did form GJs. Evident as a series of step-like transitions in both current signals that were similar in magnitude and opposite in sign, coupling was detected within as little as one second of bringing two spherical hemocytes together (Fig. 4.9A). These current steps presumably represent the opening of individual GJ channels as successive pairs of hemi-channels link up to form complete intercellular channels. The single channel conductance was calculated to be 345 pS. Three representative traces of rapid GJ formation are shown in Fig. 4.9B.

Figure 4.8 Hemocyte flattening is delayed by plating cells on lipophorin-coated glass coverslips. Thirty minutes after being plated onto untreated glass (A), hemocytes had become so extensively flattened that they could not be patch-clamped. However, when plated onto lipophorin-coated coverslips, hemocytes remained only slightly adherent to the glass for 30 min (B) or more, and were easily patch-clamped. When discoid hemocytes (arrowheads) were manipulated into contact GJ formation was not detected, but spherical or flattening cells (arrows), when pushed together, did couple. Bar=10 µm.

Figure 4.9 Recordings of single channel activity during GJ formation in cells manipulated into contact. (A) Current traces (I_1 and I_2) from both cells are shown. Cells were held at constant holding potentials of -55 and -40 mV (V_j=-15 mV). Step-like current transitions are evident, starting 8 seconds after the cells make contact (arrow). One channel opens and then closes and then many channels open as the steps sum on top of each other. That the steps are equal in magnitude and opposite in polarity in both cells is evidence that these current patterns represent GJ channel openings. The first channel opening had a conductance of about 350 pS (dotted lines on upper trace). Some of the subsequent transitions clearly had smaller conductances which may represent channel subconductances. (B) Representative current traces of GJ formation in 3 different cell pairs (#2 is the upper trace in (A)). Trace #1 shows the most rapid formation seen, with the first channel forming about 1 second after cell contact. Full details are to be found in Churchill, Coodin, Shivers, and Caveney (1993).


A
V_1 = -55 mV
V_2 = -40 mV
350 pS
I_1
I_2
10 pA
5 sec


B
#1
#2
#3
Conductance (nS)
Time After Contact (seconds)

4.4 Discussion

Hemocytes participating in the encapsulation of foreign objects *in vivo* are known to form GJs soon after being added to a capsule (Baerwald, 1975; Norton and Vinson, 1977; Caveney and Berdan, 1982; Han and Gupta, 1989; Chang *et al.*, 1991). In this study I demonstrate that hemocytes form GJs rapidly *in vitro*. E-face GJal plaques were seen in freeze-fracture replicas of hemocyte aggregates within 5 min of bleeding. That the GJs seen in freeze-fracture represent functional GJs was confirmed using a sensitive dye-transfer assay. Carboxyfluorescein passage was detected between *P. americana* hemocytes within 3 min of bleeding. It was also discovered that hemocytes derived from distantly-related insects can form functional GJs with one another *in vitro*. A quantitative CFDA assay was developed and used to study the effects of trypsin on dye-coupling between hemocytes. Finally, using lipophorin to prevent the extreme hemocyte flattening *in vitro* which precludes patch-clamping of these cells, Dennis Churchill and I have been able to use this technique to study the properties of single GJal channels as they form (Churchill *et al.*, 1993).

4.4.1 *De novo* formation of GJal structures *in vitro*

Streams of E-face particles apparently coalescing to form GJal plaques were seen on hemocytes incubated in saline for 5 min prior to fixation. The absence of clusters of E-face particles on the plasma membrane of hemocytes bled directly

into fixative indicates that hemocytes in circulation do not have pre-formed junctions. Plaques with more densely packed intramembranous particles are visible on hemocytes fixed 10 min post-bleeding. These results are consistent with a model of GJ formation based on studies of GJ re-formation by re-aggregated Novikoff hepatoma cells (Johnson *et al.*, 1989). According to this model, GJ formation begins by the docking of one (or a few) connexons from each of two cells. This nascent GJ acts as a focal point which facilitates the linking of more connexons from the two cells. Eventually a GJal plaque consisting of tightly packed connexons (and perhaps other proteins) is formed.

Strings of P-face particles which I observed on *P. americana* hemocytes bled directly into fixative were not detected by Baerwald (1974). These P-face strings were more numerous and longer on hemocytes incubated in saline prior to fixation. Discontinuous linear arrays of P-face particles, similar in appearance to those seen on hemocytes, have been observed in insect tissues that possess "barrier systems" (e.g. blood-eye, blood-testis)(Lane *et al*, 1977). It has been proposed that in these systems such tight junction-like structures might play an occluding role (Lane and Skaer, 1980). If formed by hemocytes involved in the encapsulation of a foreign object, such P-face strings might reduce or completely cut off a parasite's access to nutrients in the hemolymph.

4.4.2 Functional coupling between hemocytes *in vitro*

CFDA has been used by others to study GJα1 coupling between mouse blastomeres (Goodall and Johnson, 1982; Kidder *et al.*, 1987). There are several advantages to using CFDA over dye-injection: 1/ it is mechanically noninvasive, 2/ it allows for an accurate measurement of the time to onset of coupling, and 3/ many cells can be simultaneously labelled and observed within a single field of view in the microscope. The formation of functional GJs between hemocytes *in vitro* was detected within 3 min post-bleeding. Even though dye-transfer was detected within 3 min of adding unlabelled cells, it was apparent that the time required for GJ formation was considerably less than could be measured by this assay. This is because it takes time for the unlabelled hemocytes to settle onto the coverslip (0-10 min), and for sufficient dye to pass to an unlabelled cell to make it detectably fluorescent. The use of the double whole-cell patch-clamp technique to obtain a more accurate measurement of the time between hemocyte contact and GJ formation and to examine the properties of single channels is discussed below (Churchill *et al*, 1993).

Although hemocyte classification is controversial (Rowley and Ratcliffe, 1981; Brehelin and Zachary, 1986; Gupta, 1991b), there is growing evidence that different hemocyte types interact during cellular immune reactions in insects (Ratcliffe *et al.*, 1984; Huxham and Lackie, 1988; Anggraeni and Ratcliffe, 1991). Dye-passage to all cells within large

groups of hemocytes during CFDA assays suggests that all (or nearly all) types of hemocytes are capable of forming GJs.

Epstein and Gilula (1977) demonstrated in dye-injection experiments that functional GJs can form between cells taken from related insect species, but not from species in different orders, in co-culture. I have extended these findings to show that hemocytes from distantly related insect orders (Dictyoptera (cockroach) and Lepidoptera (moth)) form functional GJs. Although no insect GJ gene has yet been sequenced, these results indicate that there is sufficient conservation of (at least one) GJ protein among insects to enable inter-order coupling to occur.

By modifying the original CFDA assay slightly it was possible to obtain quantitative data regarding the rate at which GJal coupling is established. Using this assay I found that trypsin reduced the formation of functional GJal channels between hemocytes, but only if the surfaces of both the 'dye-donor' and the 'dye-recipient' cells were trypsinized. One explanation for these results is that trypsin cleaves GJ protein on the hemocyte surface, but that the formation of a functional channel requires that the hemi-channel on only one cell be intact. An alternative interpretation is that trypsin cleaves other proteins on the hemocyte plasma membrane, perhaps cell adhesion molecules, needed in order for GJs to form (see section 4.4.4). A third explanation is that trypsin reduces coupling via a more indirect method (e.g. by causing an increase in intracellular Ca^{2+} levels).

4.4.3 Single GJ channel formation between hemocytes

Coupling between hemocytes within 1 second of contact is faster than that seen in similar studies of GJ formation using other cell types (0.5-15 minutes)(Loewenstein *et al.*, 1978; Chow and Young, 1987; Rook *et al.*, 1988). As observed in these other patch-clamp studies, GJ formation by hemocytes proceeds by the addition of channels one-channel-at-a-time to a growing junction.

A single channel conductance of 345 pS was obtained for hemocyte GJs. This is large when compared with the single channel conductance seen in the vertebrate cell types studied to date, most of which are reported to be below 100 pS (e.g. Neyton and Trautmann, 1985), with only two in the 200-300 pS range (Chen and DeHaan, 1992; Miller et al., 1992). The 345 pS hemocyte GJ channel, however, is in close agreement with recently published values for beetle epidermal cells (Churchill and Caveney, 1993) and a mosquito cell-line (Bukauskas and Weingart, 1993). The generally larger single channel conductances of insect versus vertebrate cells is consistent with the finding of larger pore sizes in insects (Schwarzmann et al., 1981).

Given the broad range of single channel conductances observed (note different step sizes in Fig. 4.9A), the possibility exists that hemocyte GJa1 channels have distinct subconductances. Alternatively, multiple channels with different conductances may be present. Both of these possibilities have been considered to explain multiple

conductances found in GJ channels in other cells (Neyton and Trautmann, 1985; Veenstra and DeHaan, 1988; Somogyi and Kolb, 1988).

4.4.4 Mechanism of GJ formation

Hemocytes circulating *in vivo* presumably contain a pool of GJ protein, since hemocyte GJ formation occurs *in vitro* within 3 min post-bleeding. GJ precursors or complete hemi-channels might be dispersed in the plasma membrane of circulating hemocytes and/or stored in the membranes of cytoplasmic vesicles which rapidly fuse with the plasma membrane upon hemocyte activation. Adhesion of hemocytes to a solid substrate clearly is not required for GJ formation to occur since large, free-floating hemocyte aggregates form GJs *in vitro* (freeze-fracture results).

What activates circulating discoid hemocytes to become mutually adhesive and capable of flattening and forming GJs rapidly? Hemocyte activation might be initiated by wound factors released during bleeding from damaged epidermal cells at the puncture site (Cherbas, 1973). Activation might involve hemocyte aggregation, with sustained cell-to-cell contact sufficient to induce GJ formation. We, however, did not see GJ formation when discoid hemocytes were brought into contact - discoid being the morphology of inactivated circulating cells (Lackie *et al.*, 1985) - suggesting that more than cell-to-cell contact is required to induce GJ formation. Similarly in mammalian lymphocytes, cell contact alone was not

sufficient to induce GJ formation; the cells first had to be activated by a soluble mitogen (Hulser and Peters, 1972; Oliveira-Castro *et al.*, 1973).

It has been proposed that in order for the plasma membranes of two cells to come into close enough contact for GJal hemi-channels to dock, that other specific cell contacts must form first (Edelman, 1988). The necessity for the binding of Ca^{2+}-dependent cell adhesion molecules (cadherins) between cells prior to GJ formation has been demonstrated for mouse epidermal cells (Jongen *et al.*, 1991) and mouse S180 sarcoma cells (Mege *et al.*, 1988), but not for mouse blastomeres (Goodall, 1986). Hemocyte GJ formation in the absence of Ca^{2+} does not support the proposal that cadherin-like binding is a prerequisite for GJ formation during hemocyte encapsulation (Gupta, 1991a). The involvement of Ca^{2+}-independent cell adhesion molecules in hemocyte adhesion and/or GJ formation, however, cannot be ruled out.

4.4.5 What role(s) might hemocyte GJ formation play *in vivo*?

Extensive GJal coupling between hemocytes within capsules has been demonstrated in both structural (Baerwald, 1975; Norton and Vinson, 1977; Han and Gupta, 1989: Chang *et al*, 1991) and functional studies (Caveney and Berdan, 1982). Since only cells on the outer surface of a capsule have direct access to the hemolymph, junctional coupling could allow for the transfer of required metabolites to hemocytes in the inner layers of a capsule, a function for GJs that has been proposed

for other tissues (e.g. Gilula et *al.*, 1978).

GJs may also be involved in the regulation of capsule thickness (Norton and Vinson, 1977). This is supported by three pieces of evidence: 1/ soon after new hemocytes contact a capsule they form GJs with it (Baerwald, 1975), 2/ the outermost hemocytes of a capsule are able to detach and re-enter the circulation (Grimstone *et al.*, 1967; Gotz, 1986), 3/ during CFDA experiments, hemocytes were seen to form GJs and then detach as they moved apart from one another minutes later. Soon after joining a capsule, hemocytes form GJs through which they could receive a signal responsible for regulating capsule thickness.

Although hemocyte encapsulation is known to occur in many invertebrates, including arthropods, molluscs, and annelids (Gotz, 1986), only in insects has the formation of GJs during encapsulation been demonstrated. How widespread is the phenomenon of hemocyte GJ formation during encapsulation? Establishing this would indicate not only when the ability for hemocytes to form GJs evolved, but might also support or detract from the roles for hemocyte GJs proposed above.

Hemocytes are intimately involved in the repair of various insect tissues, including epidermis and central nervous system, following experimental wounding (Lackie, 1988b). During epidermal wound repair, hemocytes form a sheath over the wound upon which epidermal cells then migrate to reestablish, within 24 hours of wounding, a complete monolayer (Rowley and Ratcliffe, 1978). This suggests that cell-to-cell

signalling between hemocytes and other tissues might be important during the wound healing process. Since GJs might participate in inter-tissue signalling, it would be worthwhile to examine whether hemocytes can form GJs with cells from other tissues, such as the epidermis.

SUMMARY

Inhibition of hemocyte-substrate adhesion

1. A component in cockroach plasma inhibited hemocyte-substrate adhesion *in vitro*. Preliminary characterization indicated that it has a molecular weight >100 kDa. This component was stable at -20°C and 70°C, but not at 100°C, suggesting that it is a protein.

2. Hemocytes resuspended in purified lipophorin, either in the presence or absence of Ca^{2+}, remained non-adherent to coverslips and retained a discoid morphology *in vitro* for at least 30 min. In contrast, hemocytes incubated either with or without Ca^{2+} for 30 min in lipophorin-deficient plasma, bovine serum albumin, or saline alone adhered and flattened onto glass coverslips.

3. Calf serum inhibited hemocyte-substrate adhesion *in vitro* in the presence and absence of Ca^{2+}.

4. Human plasma proteins and IgG were tested for the ability to inhibit hemocyte adhesion. Both LDL and VLDL strongly inhibited hemocyte adhesion, however a higher concentration of VLDL was required to achieve maximum inhibition. HDL and IgG did not significantly inhibit hemocyte adhesion. These results, in conjunction with the knowledge that apoB-100 makes up approximately 90%, 54%, and 2% of the apolipoprotein

content of LDL, VLDL and HDL, respectively suggested that apoB-100 was responsible for inhibiting hemocyte adhesion.

5. Hemocyte adhesion was inhibited by apoB-100 (i.e purified LDL with an apolipoprotein content of >99% apoB-100), but not by apoA-I, apoA-II, apoC-I, apoC-II, or apoE.

6. Of eight synthetic peptides with sequences corresponding to short regions (15 to 43 amino acids) of human apoB-100, one (4154-4189) inhibited hemocyte adhesion.

7. Human apoB-89, a truncated form of apoB-100 lacking the region containing 4154-4189, also inhibited hemocyte adhesion. This indicates that, in addition to 4154-4189, one or more additional sites exist on apoB-89 which are active in inhibiting hemocyte adhesion.

GJ formation between hemocytes *in vitro*

8. Hemocytes formed E-face GJa1 plaques *in vitro* within 5 min post-bleeding.

9. A CFDA dye-transfer assay was used to confirm that the GJs formed between hemocytes *in vitro* were functional and could form in under 3 min. This strongly suggests that hemocytes circulating *in vivo* contain a pool of GJ protein, stored either in the plasma membrane and/or stored in the membranes of cytoplasmic vesicles which rapidly fuse with the plasma

membrane upon hemocyte activation.

10. Dye-coupling was detected in the absence of Ca^{2+}, indicating that involvement of Ca^{2+}-dependent adhesion molecules is not a prerequisite of GJ formation in hemocytes.

11. Dye-passage between hemocytes from distantly related insects (cockroach and moth) suggests sequence homology among GJ proteins in insects.

12. A quantitative dye-transfer assay was developed to measure the rate of GJ formation between hemocytes. It was determined that trypsin reduced the formation of functional GJal channels between hemocytes, but only if the surfaces of both the 'dye-donor' and 'dye-recipient' cells were trypsinized. This could be due to the cleaving of GJ protein or cell adhesion molecules on the hemocyte surface.

13. Hemocyte flattening *in vitro* was delayed by plating cells on lipophorin-coated glass coverslips, thereby facilitating patch-clamp studies of these cells.

14. Double whole-cell voltage-clamp was used to measure GJ formation between hemocytes which were pushed together. Electrical coupling was detected within one second of cell-cell contact, and the single channel conductance was calculated to be ≈345 pS.

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