THE INVOLVEMENT OF $G\alpha_S$ PROTEINS AND INTEGRINS IN THE CELLULAR IMMUNE RESPONSES OF A LEPIDOPTERAN INSECT, *Galleria mellonella*

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A thesis submitted to the Graduate and Postdoctoral
Studies Office in partial fulfillment of the requirements
for the Degree of Master of Science

ABSTRACT

The greater wax moth, *Galleria mellonella*, is used extensively as a model to study innate immunity. The multicellular process of nodulation acts to isolate large numbers of small microbes (bacteria and yeasts) in the open circulatory system (hemocoel) of the insect and is directed by the main immune blood cell (hemocyte) types, the granular cells and the plasmatocytes. G proteins and their downstream products have been implicated in both early (hemocyte-microbe adhesion) and late (humoral) immune responses, however, their involvement in hemocyte-hemocyte interactions leading to nodule formation is not known. Herein, I treated hemocytes with an AB_5 protein, whole cholera toxin (CTX), known to stimulate the $G\alpha_s$ subunit of heterotrimeric G proteins and is responsible for activating adenylate cyclase and elevating intracellular cAMP in mammalian neutrophils. CTX caused a bimodal increase in hemocytes per *in vitro* microaggregate and nodule frequency *in vivo*, wherein increasing high cholera toxin concentrations induced these effects, while low concentrations induced levels of hemocytes per *in vitro* microaggregate and nodule frequency similar to the highest concentration of CTX, but the hemocytes showed reduced substratum-related adhesion. CTX-dependent bacterial (*Bacillus subtilis*) removal from the insect hemocoel was inversely correlated with nodule frequency. The cholera toxin B-subunit was responsible for the effects of CTX at higher concentrations, while isolated cholera toxin A-subunit had no effect on the hemocytes. *In vitro* CTX-induced, hemocyte microaggregate formation was inhibited by RGD peptides, suggesting the involvement of integrins.

Human integrin antibodies to α_v, β_3, α_5, and β_1 bound integrin-like molecules from unseparated, whole hemocyte lysates and labeled both the granular cells and plasmatocytes adhering to coverslips. The anti-α_5 integrin labeled the junction between interacting granular cells and plasmatocytes, the frequency of this labeling pattern increasing with CTX treatment. Only anti-α_5 and -β_1 prevented *Ba. subtilis* removal from the insect hemocoel with increasing antibody concentration.

RÉSUMÉ

La fausse teigne de la cire, *Galleria mellonella*, est utilisée extensivement comme modèle pour etudier le système immunitaire inné. Le processus multicellulaire de nodulation isole de grandes quantités de microbes de petite taille (bacteries et levures) dans l'appareil circulatoire (hémocèle) de l'insecte et est controlé par les deux cellules sanguines immunitaires (hémocytes) principales: les cellules granulaires et les plasmatocytes. Les protéines G et leurs produits de réactions sont impliqués dans les réponses immunitaires precoces (l'adhésion hémocytes-microbes) et tardives (humorale), cependant, leur participation dans les intéractions hémocytes-hémocytes conduisant à la formation des nodules demeure inconnue. Ci-dessous, j'ai traité les hémocytes avec la protéine AB_5, la toxine cholérique (CTX), reconnue pour sa stimulation des $G\alpha_s$, une sous-unité des protéines G hétérotrimériques et est responsable de l'activation de l'adénylate cyclase et de l'augmentation de l'AMP cyclique intracellulaire dans les neutrophiles mammifère. La CTX a causé une augmentation bimodale du nombre d'hémocytes par micro-agrégats *in vitro* et de la fréquence des nodules *in vivo*. L'augmentation des concentrations élevées a induit une augmentation de ces paramètres, tandis que de faibles concentrations induit des taux d'hémocytes par micro-agrégats *in vitro* et de la fréquence de nodules *in vivo* semblables à ceux obtenus avec la concentration la plus élevée de CTX, bien que les hémocytes aient démontré une réduction d'adhérence au substrat. Le retrait CTX-dépendantes des bacteries (*Bacillus subtilis*) injecter dans l' hémocèle de l'insecte était inversement corrélé avec la fréquence des nodules. La sous-unité B de la CTX était responsable des effets obtenus aux concentrations plus élevées, alors que la sous-unité A de la CTX n'a eu aucune effet sur les hémocytes. La formation des micro-agrégats *in vitro* par la CTX était inhibée par des peptides RGD, suggérant l'implication des intégrines.

Les anticorps humains contre les sous-unités intégrines α_v, β_3, α_5, et β_1 ont lié des molécules provenant d'hémocytes entiers lysés et ont lié les cellules granulaires et les plasmatocytes adhérés aux lamelles. L'anti-α_5 intégrine a lié la jonction entre les cellules granulaires interagissant avec les plasmatocytes, la fréquence de cette liasion augmentant avec la concentration de la CTX. En outre, seuls l'anti-α_5 et -β_1 intégrines ont empêché le

retrait de *Ba. subtilis* de l'hémocèle de l'insecte avec l'augmentation de la concentration d'anticorps.

CONTRIBUTION OF AUTHORS

The author of this thesis has received guidance and has held discussions with Professors Craig A. Mandato and Gary B. Dunphy. Professor Gary B. Dunphy assisted with *in vivo* experiments with the cholera toxin B-subunit. Bama Dayanandan provided answers to questions about western blotting and immunofluorescence labeling protocols. Tong Zhang and Drew Williams provided guidance with respect to immunofluorescence imaging. In all other instances, the experimental designs and collection of data were carried out by the author. Professors Craig A. Mandato and Gary B. Dunphy corrected and edited the thesis.

ACKNOWLEDGEMENTS

‘The miracle, or the power, that elevates the few is to be found in their industry, application, and perseverance under the prompting of a brave, determined spirit (Mark Twain)’. I’ve worked hard for this degree, but without the relentless encouragement and guidance of my supervisors, Drs. Gary B. Dunphy and Craig A. Mandato, I wouldn’t be writing this thesis. They’ve helped me grow as a researcher in these past years, and I can safely say that my qualities as a researcher [equal parts enthused, perserverent, curious, and skeptic (w/v)] are thanks to them. I’m grateful for their generosity and help with honing my scientific skills. Thank you both for influencing me so much. I have stood on the shoulders of giants.

Many thanks to past and present members of my lab and neighboring labs for their assistance, insightful conversations, and friendship: Paschalis Giannoulis, Ndonkeu Walter, Khash Afhsar, Gitanjali Arya, Jon Pritchard, Emily Waters, Oliver Trottier, Michelle Poily, Kristin Radtke, Bama Dayanandan, Drew Williams, Tong Zhang, Elizabeth Lawrence, Osama Gharib, and Iain Geoffrey. Thanks to Marlene Parkinson for her kindness and administrative super-powers. I’d like to thank the members of my graduate committee, Drs. Elaine Davis and Jean-Francois Cloutier, and my mentor, Dr. Carlos Morales.

I’m very appreciative of my family who’ve provided me with support and unconditional love throughout my degree: thank you Cloe Lapointe, Guylaine Morin, and Martial Lapointe. An extra thanks to these people for tolerating me throughout this time and their perpetual encouragement with questions largely related to: ‘So when are you finishing?’

I’d like to thank the following people outside of university for their support, friendship, and patience: Shanna Dupuis, Mathieu Biard, Steve Cyr, Ryan Curry, Mathieu Gagnon, Meghan Lambertus, Mariane Morin, Maxime Morin, David Valerio, and Amanda Lindeman. Thank you for giving me something else to think about. I’m grateful to Casey Crescenzo, Matt Joynt, Jesse Lacie, and Owen Brinley for having gotten me through long bus rides. And for that matter Stephen Baxter, William Gibson, Herodotus, and Thucydides for their captivating literary works. I’d also like to thank Claude Vinet of

Miel Claude Vinet (Vaudreuil-Dorion, Quebec) for the generous supply of brood comb, and also for financial support from NSERC making this work possible.

This degree means a lot to me.

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LIST OF ABBREVIATIONS

AMP: antimicrobial peptide

ALP III: apolipophorin III

BSA: bovine serum albumin

CTX: whole cholera toxin

CTB: cholera toxin B-subunit

CTA: cholera toxin A-subunit

cAMP: cyclic adenosine monophosphate

ECM: extracellular matrix

EDTA: ethylenediaminetetraacetic acid

ERK: extracellular receptor kinase

FAD: flavin adenine dinucleotide

FAK: focal adhesion kinase

fMLP: *N*-formylmethionyl-leucyl-phenylalanine

GPCR: G protein-coupled receptor

GTPase: guanine triphosphate hydrolase

IgG: immunoglobulin

Imd: Immunodeficiency

JNK: c-Jun N-terminal kinase

LPS: lipopolysaccharide

LTA: lipoteichoic acid

MAPK: mitogen-activated protein kinase

NADPH: nicotinamide adenine dinucleotide phosphate

PAMP: pathogen associated molecular pattern

PBS: phosphate-buffered saline

PG: prostaglandin

PKA: cAMP-dependent protein kinase

PKC: calcium-dependent protein kinase

PLA: phospholipase A

PO: phenoloxidase

PRR: pattern recognition receptor

PTX: pertussis toxin

RGD(S): sequence of amino acids Arginine – Glycine – Aspartic acid (– Serine)

RGE(S): sequence of amino acids Arginine – Glycine – Glutamic acid (– Serine)

TBS: tris-buffered saline

LIST OF COMMON AND LATIN NAMES OF CITED INVERTEBRATE ANIMALS

PHYLUM: PORIFERA

O. tennis: Sponge, *Ophlitaspongia tennis*

PHYLUM: CNIDARIA

A. millepora: Staghorn coral, *Acropora millepora*

PHYLUM: NEMATODA

Order: Rhabditidae

C. elegans: Nematode, *Caenorhabditis elegans*

St. carpocapsae: Entomopathogenic nematode, *Steinernema carpocapsae*

PHYLUM: TREMATODA

Order: Strigeidida

Sc. mansoni: Trematode, *Schistosome mansoni*

PHYLUM: ARTHROPODA

Class: Insecta

Order: Blattaria

Gr. portentosa: Madagascar hissing cockroach, *Gromphadorhina portentosa*

Pe. americana: American cockroach, *Periplaneta Americana*

Order: Coleoptera

Tr. castaneum: Red flour beetle, *Tribolium castaneum*

T. molitor: Mealworm beetle, *Tenebrio molitor*

Z. morio: Darkling beetle, *Zophobas morio*

Order: Diptera

An. albimanus: Mosquito, *Anopheles albimanus*

An. gambiae: Mosquito, *Anopheles gambiae*

Ce. capitata: Medfly, *Ceratitis capitata*

D. melanogaster: Fruit fly, *Drosophila melanogaster*

Order: Hemipteran

R. prolixus: Triatomid bug, *Rhodnius prolixus*

Order: Hymenoptera

Bo. terrestris: Buff-tailed Bumblebee, *Bombus terrestris*

Order: Lepidoptera

Ant. polyphemus: Polyphemus moth, *Antheraea polyphemus*

Ag. urticae: Small tortoiseshell, *Aglais urticae*

Bom. mori: Silkworm, *Bombyx mori*

Ep. kuehniella: Mediterranean flour moth, *Ephestia kuehniella*

G. mellonella: Wax moth, *Galleria mellonella*

H. cecropia: Cecropia moth, *Hyalophora cecropia*

Ma. sexta: Tobacco hornworm, *Manduca sexta*

Mal. disstria: Forest tent caterpillar, *Malacosoma disstria*

Pi. brassicae: Cabbage butterfly, *Pieris brassicae*

Ps. includens: Soybean looper, *Pseudoplusia includens*

Pse. separata: Rice armyworm, *Pseudaletia separata*

Pse. unipuncta: True armyworm, *Pseudaletia unipuncta*

Sa. cynthia: Ailanthus silkmoth, *Samia cynthia*

Sp. exigua: Beet armyworm, *Spodoptera exigua*

Sp. littoralis: African cotton leafworm, *Spodoptera littoralis*

Order: Orthoptera

Ac. domesticus: House cricket, *Acheta domesticus*

PHYLUM: MOLLUSCA

Class: Bivalvia

Order: Ostreoida

Cr. gigas: Pacific oyster, *Crassostrea gigas*

LIST OF MICROBE TYPE AND LATIN NAMES OF UNICELLULAR PROKARYOTES AND EUKARYOTES

Ba. subtilis: Gram-positive bacterium, *Bacillus subtilis*

Be. bassiana: Entomopathogenic fungi, *Beauvaria bassiana*

B. cepacia complex: Group of Gram-begative bacteria, *Burkholderia cepacia*

Ca. albicans: Yeast, *Candida albicans*

Es. coli: Gram-negative bacterium, *Escherichia coli*

E. cloacae: Gram-negative bacterium, *Enterobacter cloacae*

E. facaelis: Gram-negative bacterium, *Enterococcus facaelis*

E. hirae: Gram-negative bacterium, *Enterococcus hirae*

F. tularensis: Gram-negative bacterium, *Franciscella tularensis*

L. pneumophila: Gram-negative bacterium, *Legionella pneumophila*

Me. anisopliae: Entomopathogenic fungi, *Metarhizium anisopliae*

M. lysodeikticus: Gram-positive bacterium, *Micrococcus lysodeiktus (= M. luteus)*

P. aeruginosa: Gram-negative bacterium, *Pseudomonas aeruginosa*

Sta. aureus: Gram-positive bacterium, *Staphylococcus aureus*

S. lactis: Gram-positive bacterium, *Streptococcus lactis*

S. pyogenes: Gram-positive bacterium, *Streptococcus pyogenes*

X. nematophila: Entomopathogenic Gram-negative bacterium, *Xenorhabdus nematophila*

Y. pseudotuberculosis: Gram-negative bacterium, *Yersinia pseudotuberculosis*

CHAPTER 1: Literature Review and Thesis Introduction

LITERATURE REVIEW

The following is a review of the literature concerning the processes involved in an insect immune response. I have provided comparisons between arthropod orders and other phyla, including chordates, as a means to fill in gaps in the field but to also present the consistency of the metazoan immune response. I begin by briefly introducing my animal model, *Galleria mellonella*, and proceed by describing the steps taken by the insect's immune system in quarantining an infection, beginning with microbial infiltration into the circulation system and ending with microbial removal and killing by the insect's immune system.

EXPERIMENTAL ANIMAL

Galleria mellonella

The greater wax moth is an economically important lepidopteran pest insect that is a major financial concern to beekeepers, the insect infesting and collapsing bee colonies by tunneling through honeycomb and consuming wax and honey (Singh, 1962; Covell, 1984; Nielson and Brister, 1979; Eischen *et al*., 1986). Due to the ease of rearing, the insect does provide commercial benefits, such as its use as fish bait and reptilian and bird food (Mohamed and Coppel, 1983). With the availability of mass-rearing techniques and post-transcriptional machinery similar to mammals, insects provide a cost-effective means of expressing commercially-important proteins (Markaki *et al.*, 2007; Tsuchiya *et al.*, 2009).

G. mellonella is holometabolous displaying four life stages: egg, larvae, pupa, and adult. An adult moth can produce upwards of 150 eggs often bound together and laid in crevices around the beehive (Singh, 1962). Eggs hatch 3 – 4 days later emerging as pale yellow larvae measuring ~1 mm after which they experience discontinuous growth through seven instar (intermolt) stages (Wani *et al*., 1997), the latter determined by weight and head capsule width. Last instar larvae spin a cocoon and pupate after which a beige-molted adult moth emerges. The complete life span of the insect will range between one to six months (Marston *et al*., 1975).

Ready rearing and extensive characterization of the cellular immune responses of *G. mellonella* make it an ideal research tool in studying host responses to human pathogens. Many physiological and biochemical processes occurring in insect blood cells (hemocytes) are similar in higher animals. For example, antibodies raised against human neutrophil *phox* proteins, responsible for superoxide production and microbial killing, label also proteins of similar molecular weights and function in *G. mellonella* hemocytes (Bergin *et al.*, 2005; Renwick *et al.*, 2007). Human antibodies to tumour necrosis factor and interleukin 1, molecules participating in immune signaling and responsible for leukocyte behaviour during infection, will identify also molecules on *G. mellonella* hemocytes (Wittwer *et al.*, 1999). With comparable molecules and mechanisms mediating immunocyte behaviour in both insects and mammals, it is not surprising that human pathogens can parasitize insects, justifying the use of insects as models for host-human pathogen interactions (Vilcinskas, 2010).

Insects are commonly used to study the effect of drugs, such as anti-microbial medication, on the clearance of infection (Kemp and Massey, 2007). *G. mellonella* is susceptible to human pathogens, showing similar mortality rates to the human pathogenic fungus, *Candida alicans* in murine models (Brennan *et al.*, 2002; Dunphy *et al.*, 2003). The causative agent of tularemia, *Franciscella tularensis*, is an intracellular parasite of human macrophages that can parasitize also *G. mellonella* hemocytes and kill the host insect (Aperis *et al.*, 2007). Mutant strains of the human pathogen *Pseudomonas aeruginosa* show a positive correlation between increasing LD_{50} in *G. mellonella* and mouse survivability (Jander *et al.*, 2000), and the degree of virulence has been associated with similar virulence genes (Choi *et al.*, 2002). *G. mellonella* are also susceptible to infection by *Yersinia pseudotuberculosis* (responsible for tuberculosis-like symptoms in animals), which is able to live intracellularly in hemocytes and kill the larvae (Champion *et al.*, 2009). The *Burkholderia cepacia* complex, a family of Gram-negative bacteria, some of which are responsible for pneumonia in humans, can infect *G. mellonella*, the degree of virulence being similar to rats (Seed and Dennis, 2008). Extracellular gelatinase from *Enterococcus facaelis*, typically a hospital-acquired pathogen, inhibits humoral immune molecules from *G. mellonella* and humans (Park *et al.*, 2007). Drug companies may use insects as an effective means to test drugs before progressing to more expensive

animal trials, and *G. mellonella* as a host for human pathogens and/or pharmaceutical-test animal has been recently reviewed by Vilcinskas (2010). Also, hemolymph-isolated molecules, such as alloferons and antimicrobial peptides, have potential use as anti-viral and anti-cancer agents (Chernysh *et al.*, 2002; Papo and Shai, 2005).

IMMUNITY

Infection route

Microorganisms can infect insects by surpassing their structural/physical barriers, such as the cuticle and gut epithelium (Bulla *et al.*, 1975). Most commonly, microbes can infect an insect by way of its oral cavity, entering the insect during meals and later penetrating its gut epithelium to enter the hemocoel (Bulla *et al.*, 1975). Other microbial parasites can bypass the insect's integument by digesting the structural components of the cuticle, such as chitin, or can enter the hemocoel from wounds caused by mechanical injury or other parasites (Bulla *et al.*, 1975). The hemocoel of the insect contains the fluid hemolymph, which has both the characteristics of mammalian blood and the lymphatic system (Gullan and Cranston, 2005). The insect has an open circulatory system where the hemolymph flows freely around all organs, transports nutrients, removes metabolites, and endows the insect with immune protection against infectious microbes (Gullan and Cranston, 2005).

Insect immunity

The insect immune responses are coordinated by blood cells (hemocytes) in the hemolymph and the surrounding fat body organ, a functional analog of the mammalian liver (Keeley, 1985). Insect immunity consists of innate immunity (Hoffman, 2003), and is comprised of germline encoded receptors (pattern recognition receptors) recognizing evolutionarily unchanged microbial antigens (pathogen-associated molecular patterns such as lipopolysaccharide, lipoteichoic, and β1,3-glucan) ultimately activating both cellular and humoral immune responses (Lemaitre and Hoffman, 2006). Unlike mammals, insects do not possess an acquired immune response, the ability to produce antibodies from antigen-stimulated, genetically-recombined lymphocyte receptors (Fearon and Locksley, 1996). The innate immune response exhibits a spectrum of activity, wherein it

provides both immediate and prolonged protection (Gillespie *et al.*, 1997; Eleftherianos *et al.*, 2006). Immediate reactions include cellular (hemocyte) responses, such as phagocytosis, nodulation, and encapsulation, and constitutive humoral plasma components acting as recognition proteins and antimicrobial agents (Gillespie *et al.*, 1997). The induced humoral immune response consists of the *de novo* synthesis of antimicrobial peptides elicited by infections and displays some specificity according to microbial species and Gram reaction type (Lemaitre and Hoffman, 2006). Where long-term immune memory, i.e. antibody production, may be biologically unfeasible for an insect, due to its short lifespan, induced immunity provides standing protection, the duration varying with insect and microbe species, against subsequent infections. *G. mellonella* larvae challenged with *Enterobacter cloacae* 17 h and 24 h after being initially vaccinated with a lower non-lethal dose of the same bacteria display higher survival rates than without vaccination (Wiesner, 1991). The american cockroache, *Periplaneta americana* immunized with dead *P. aeruginosa* display higher survival rates against subsequent injections of the same bacteria, *Micrococcus lysodeikticus*, and *Streptococcus lactis* compared to buffer controls, this immunization decreasing over a 14 day period (Faulhaber and Karp, 1992). Subsequent challenge (3 weeks after initial vaccination) of adult Buff-tailed bumblebees, *Bombus terrestris* with the same bacterial strain, bacteria of the same genus but different species, or an unrelated bacterial species results in more rapid clearance of the bacteria and higher insect survival rates than non-vaccinated insects (Sadd and Schmid-Hempel, 2006). Induced immunity appears to wane over a period of hours to weeks varying with the insect species (Wiesner, 1991; Faulhaber and Karp, 1992; Sadd and Schmid-Hempel, 2006), however, whether it provides longer-standing protection in insects with longer lifespans, such as cicadas (2 – 17 yrs), remains unclear.

Immediate innate immune protection is achieved by two tightly intervolved responses, the cellular and humoral responses (Gillespie *et al.*, 1997). The fat body organ is the predominant elicitor of the humoral response, however, hemocytes are known to participate as well (Gillespie *et al.*, 1997; Lemaitre and Hoffman, 2006). Hemocytes, whose morphology and function are usually similar between closely related insect orders (Price and Ratcliffe, 1974), are capable of quarantining microbes by way of cellular

immune reactions (described below; Gillespie *et al.*, 1997). The five hemocyte types characterized in the majority of insect species (lepidopteran, dipteran, coleopteran, and hemipteran orders) used as research models include the plasmatocytes, granular cells, oenocytoids, spherulocytes, and prohemocytes (Price and Ratcliffe, 1974). In the case of *Drosophila melanogaster* hemocytes, which contrast in terminology, the overall immune functions and processes are similar to those seen in other insect orders (Lavine and Strand, 2002). For the purpose of this review, hemocyte nomenclature will be based on the work of Price and Ratcliffe (1974).

Innate cellular immunity must differentiate microbial non-self antigens (exogenous microorganisms and their products) from normal self antigens (endogenous cells and tissues) to stage an immune response (Medzhitov and Janeway, 1997). Of the five hemocyte types, only the plasmatocytes and granular cells are predominantly involved in the cellular immune responses (Lavine and Strand, 2002), and therefore in this thesis emphasis will be placed on them. The phase bright, spherical granular cells are the immune-surveyors of the hemocoel and are the first to respond to an infection (Ratcliffe and Gagen, 1977), whereas the phase dark, amoeboid plasmatocytes are secondary responders activated by granular cell stimulation (Strand and Clark, 1999; Nardi *et al.*, 2005). The spherulocytes may be involved in maintaining the integrity of basement membranes of surrounding tissues as they transport some of the necessary components (Sass *et al.*, 1994), while the oenocytoids, which synthesize the phenoloxidase zymogen, are implicated in the melanization reaction (see section *Phenoloxidase*; Iwama and Ashida, 1986). The prohemocytes, originating from the hematopoietic imaginal discs, may participate in plasmatocyte, but not granular cell, differentiation in the American cockroach, *Pe. americana*, the mealworm beetle, *Tenebrio molitor*, and two lepidopterans, *Galleria mellonella* and *Manduca sexta* (Gupta and Sutherlan, 1966; Beaulaton, 1979; Nardi *et al.*, 2003). However, prohemocytes in the lepidopteran silkworm, *Bombyx mori*, have been observed differentiating into both plasmatocyte and granular cell lineages (Yamashita and Iwabuchi, 2001). This literature review will introduce and discuss the immune responses and describe the involvement of granular cells and plasmatocytes, predominantly in lepidoptera, during these responses as a function of time from the moment the microbe enters the hemocoel.

Immune recognition

Once a microbe enters the circulation system of the insect, it is recognized by plasma/humoral (opsonins) and hemocyte-associated factors. This is important since many insect pathogens and parasites have evolved strategies to evade immune recognition to prevent the insect from mounting an effective immune response (Beauvais *et al.*, 1989; Brivio *et al.*, 2002; Walter *et al.*, 2008). Microbes are recognized by pattern recognition receptors (PRRs) binding to pathogen-associated molecular patterns (PAMPs). The humoral protein lysozyme is capable of binding to microbial surfaces (Lee, 2007) which normally damages the surfaces rendering them more susceptible to hemocytes (Dunphy *et al*., 1986; Dunphy and Webster, 1991). Soluble C-type lectins, such as *Ma. sexta* immunlectin-4 and *Bom. mori* multibinding protein (BmMBP) and lipopolysaccharide-binding protein (BmLBP), can bind to bacterial and fungal surfaces and enhance cellular immune responses, such as adhesion, microaggregation, nodulation, and melanization (Koizumi *et al.*, 1999; Watanabe *et al.*, 2006; Yu *et al.*, 2006). Noduler, also a C-type lectin, can form cell coacervates by binding to microbial and hemocyte surfaces ultimately leading to nodulation (Gandhe *et al.*, 2007). A lectin identified on the surface of *G. mellonella* plasmatocytes binds fungal β-1,3-glucans, but its functions are not known (Matha *et al.*, 1990). Plasma lectins typically recognize carbohydrates on microbial molecular antigens or after removal by enzymatic treatment, both events enhancing hemocyte activity (Dunphy *et al.*, 1986). Hemolin, a member of the immunoglobulin superfamily, binds to lipoteichoic acid (LTA) and the lipid A and O-side chains of lipopolysaccharide (LPS; Yu and Kanost, 2002). Inhibition of hemolin by parasites suppresses phagocytic and nodulation responses (Labropoulou *et al.*, 2008). The pentapeptide bridges between peptidoglycan strands in bacterial cell walls consist of amino acids, the most important to immune recognition being the third amino acids, e.g. either lysine (Lys) or α,ϵ-diaminopimelic acid (DAP) depending on Gram-type (Doyle and Dziarski, 2001). Soluble peptidoglycan recognition proteins (PGRP) are capable of reacting with the peptidoglycan amino acids, and depending on Gram-type, can elicit the expression of separate sets of antimicrobial peptides (Takehana *et al*., 2002; Dziarski and Gupta, 2006). The lipid-transport protein, apolipophorin III (ALP III) acts as a

multifunctional pattern recognition protein recognizing the Gram-positive and -negative bacterial antigens, LTA and LPS, respectively (Dunphy and Halwani, 1997; Halwani *et al.*, 2000), and the fungal cell wall component, β-1,3-glucan, the latter enhancing the encapsulation response (more on ALP III below; Whitten *et al.*, 2004). The β-1,3-glucan binding protein found in *Ma. sexta* hemolymph acts as an opsonin by binding to the surface of and agglutinating yeast and Gram-negative and -positive bacteria (Ma and Kanost, 2000). The *Ma. sexta* humoral protein leureptin shows comparable sequencing and functionality to the ligand binding region of mammalian Toll-like receptor 4, binding LPS and associating with hemocyte surfaces (Zhu *et al.*, 2003; Kanost and Nardi, 2010).

Divalent cations, including zinc, calcium and magnesium, are known to influence downstream insect cellular responses, such as adhesion to foreign surfaces and plasmatocyte spreading (Willott *et al.*, 2002; Willott and Tran, 2002; Zakarian *et al.*, 2003, Giannoulis *et al.*, 2005). These cations likely have an effect on opsonins responsible for bridging hemocytes and antigens, as seen with invertebrate lectins/agglutinins and similarly with mammalian complement proteins (Renwrantz and Stahmer, 1983; Tuan and Yoshino, 1987; Smith *et al.*, 1999; Benjamini *et al.*, 2000). However, invertebrate recognition proteins of the immunoglobulin superfamily, such as *Hyalophora cecropia* hemolin (Bettencourt *et al.*, 1999), act as divalent cation-independent opsonins, similar to vertebrate immunoglobulins (Bayne, 1990).

Hemocytes have also surface receptors capable of recognizing non-self material (Ehlers *et al.*, 1992). In mammals, these interactions, devoid of humoral influence, can be described as non-opsonic, wherein immunocytes can bind to adhesins on microbial surfaces (Ofek *et al.*, 1995). Epitope-based receptors include the LPS/LTA receptor(s) on *Bom. mori* hemocytes (Ohta *et al.*, 2004). However, surface recognition receptors may act also as scavenger receptors binding to similar polyanionic or polycationic antigen moieties, such as the LTA/LPS scavenger receptor found on *Spodoptera exigua* hemocytes (Costa *et al.*, 2005). Furthermore, plasma-free hemocytes recognize foreign surfaces with distinct hydrophobic and electrostatic properties (Dunphy and Nolan, 1980; Lackie, 1983; Ehlers *et al.*, 1992), as in mammalian blood cells (Ofek *et al.*, 1995; Pollard *et al.*, 2001), suggesting promiscuous receptors (Dettloff *et al.*, 2001) but this is not clear. This does not preclude the same receptors recognizing both physicochemical

properties and epitopes. Some of the aforementioned invertebrate humoral PRRs have enzymatic properties which could digest microbial antigens producing fragments that trigger an immune response, whereas some, once bound to its PAMP, may bind and cluster hemocyte receptors to activate a cellular immune response (Bayne, 1990; Kanost and Nardi, 2010), similar to mammalian LPS-binding protein and the surface receptor CD14 (Gegner *et al.*, 1995).

Early cellular immune response

Hemocyte-substratum adhesion

Adhesion, occurring after recognition, is a crucial event in the removal of a microbe from the hemocoel, without which the late cellular immune responses, phagocytosis, nodulation, and encapsulation can not occur. The initial adhesion reaction can be distinguished as a post-recognition event involving the interaction between the cell and the substratum/microbe. Immunocytes from animals, including invertebrates such as poriferans (Cheng *et al.*, 1968), cnidarians (Olano and Bigger, 2000), annelids (Porchet-Henneré *et al.*, 1987), molluscs (Sminia *et al.*, 1979), arthropods (Gupta, 1991) and fish (Overland *et al.*, 2009), respond to foreign material. In insects, adhesion molecules originate from the hemocytes (Ball *et al.*, 1987), as indicated by studies limiting plasma content wherein hemocytes are still capable of adhesion (Dunphy and Nolan, 1980; Lackie, 1983; Ehlers *et al.*, 1992). Arthropod adhesion molecules can bind foreign surfaces and stimulate hemocyte-substratum adhesion (Johansson and Söderhall, 1988; Liang *et al.*, 1992; Scapigliati *et al.*, 1997; Holmblad and Söderhall, 1999), but the identity of these adhesion proteins and their hemocyte receptors in lepidopterans are largely unknown. *G. mellonella* granular cells degranulate during interactions with *Escherichia coli* bacteria to release proteins adhering bacteria to their surface (Rowley and Ratcliffe, 1976), which resembles human neutrophil degranulation during adhesion to foreign surfaces (Wright and Gallin, 1979; Bockenstedt and Goetzl, 1980). Adhesion to a substratum involves integrin receptors in both lepidopterans and humans (Todd *et al.*, 1984; Lavine and Strand, 2003).

Integrins are surface receptors comprised of two non-covalently associated α and β subunits that provide a link between the cell and the extracellular matrix (ECM; Hynes,

2002). Several α and β integrin subunits have been identified on lepidopteran hemocytes (Lavine and Strand, 2003; Levin *et al.*, 2005; Zhuang *et al.*, 2007b; Zhuang *et al.*, 2008). The β subunits of integrins have also been identified in poriferans (*Ophlitaspongia tennis*; Brower *et al.*, 1997), cnidarians (*Acropora millepora;* Brower *et al.*, 1997*)*, nematodes (*Caenorhabditis elegans*; Gettner *et al.*, 1995), crustaceous arthropods (Holmblad *et al.*, 1997), and mammals (Hynes, 2002). The integrin α and β subunits exhibit conserved protein structures between the higher animal phyla (Holmblad *et al.*, 1997; Zhuang *et al.*, 2008). A common motif in many ECM proteins is the three amino acid sequence arginine - glycine - aspartic acid (RGD), which is recognized by integrin receptors (Ruoslahti, 1996). Soluble RGD peptides inhibit immunocyte adhesion and spreading, but promote these reactions when bound to a surface in tunicates (Ballarin *et al.*, 2002), snails (Davids and Yoshino, 1998), crayfish (Johansson and Söderhall, 1989), and insects (Pech *et al.*, 1995; Hagen and Kläger, 2001). There are also RGD-independent hemocyte-substratum interactions (Lamprou *et al.*, 2007). Despite similar signal transduction molecules (focal adhesion kinase and mitogen activated protein kinase) involved in medfly, *Ceratitis capitata* hemocyte engulfment of bacteria and latex beads, the latter is RGD-receptor independent suggesting that distinct blood cell-microbe adhesion reactions ultimately lead to phagocytosis (Lamprou *et al.*, 2007), similar to phagocytosis of apoptotic neutrophils by human fibroblasts (Hall *et al.*, 1994). We could reason that the release of different adhesion molecules from hemocytes may demonstrate a mechanism where the hemocyte adhesion response is the culmination of several responses to non-self material, given that the surface of a microbe contains a myriad of PAMPs, where individual PAMPs could stimulate separate responses. That different adhesion proteins are released depending on the type of PAMP, different cellular responses, such as phagocytosis or nodulation, may occur in response to the type of adhesion protein adhered to the foreign surface. Briefly, different concentrations of the same microbe can induce separate responses (phagocytosis or nodulation) by causing the release of separate adhesion proteins, albeit some with similar binding sequences (i.e. RGD), binding to different surface receptors. It is unclear whether the resulting adhesion protein-receptor interaction could determine the type of cellular immune response.

Several signaling factors affecting the adhesion response have been elucidated, glass slides or coverslips being used as an *in vitro* tool to measure non-self reactions (Walters, 1970; Dean *et al.*, 2004b; Giannoulis *et al.*, 2005; Marin *et al.*, 2005). Cell adhesion to cover slips has been interpreted as an *in vitro* model for phagocytosis (Swanson and Baer, 1995; Dean *et al*., 2004b) and encapsulation (Davies and Preston, 1987; Brooks and Dunphy, 2005). Plasmatocytes are the only hemocyte types that spread on artificial surfaces, whereas granular cells anchor themselves to a substratum usually without extensive spreading (Price and Ratcliffe, 1974), although granular cells from *Pseudoplusia includens* and *G. mellonella* are known to exhibit limited flattening (Chain and Anderson, 1982; Lavine and Strand, 2002). Strictly referring to *in vivo* events, hemocytes do not spread over small microorganisms during phagocytosis (Scapigliati and Mazzini, 1994), and hemocyte spreading has only been observed during the nodulation and encapsulation responses (Schmit and Ratcliffe, 1977). Whether hemocytes can spread on internal tissues is unknown. Hyperspreading hemocytes collected from bacteria-infected *Ma. sexta* appear smaller *in vivo* despite the occurrence of phagocytosed particulates inside the cell (Dean *et al.*, 2004b), implying they do not spread in the absence of large surfaces, such as coverslips. Consequently, hemocytes adhering to glass slides or coverslips likely represent an encapsulation response as this agrees with its very definition (described below). The use of drugs influencing signaling molecules have been used to increase the number of hemocytes of a given type capable of adhering to artificial surfaces (above buffer control levels), which implies the existence of subtypes of plasmatocytes and granular cells with differential sensitivity to foreign material (Giannoulis *et al.*, 2005; Marin *et al*., 2005; Gulii *et al.*, 2009).

Cyclic adenosine monophosphate (cAMP), an adenosine triphosphate derivative, is responsible for hemocyte adhesion to foreign surfaces (Marin *et al.*, 2005; Brooks and Dunphy, 2005; Gulii *et al.*, 2009). Lower levels of intracellular cAMP will increase the adhesion response to a substratum in insects, much like that seen in mammalian granulocytes (Bryant and Sutcliffe, 1974; Derian *et al*., 1995). Phosphodiesterases, including cAMP-hydrolyzing isoenzymes, will influence also hemocyte adhesion in insects (Marin *et al*., 2005), similar to neutrophil-vascular endothelium adhesion (Jones *et al.*, 2005). The downstream effectors of cAMP also affect insect hemocytes by increasing

intracellular protein kinase (PK) A which decreases hemocyte adhesion to bacteria and glass (Zakarian *et al.*, 2003; Brooks and Dunphy, 2005). The opposite occurs in molluscan hemocytes (Chen and Bayne, 1995b), however, this may be a product of the modulating-drug concentration. cAMP has a bimodal effect on insect hemocyte adhesion depending on its intracellular concentration, whereby lower levels increase hemocyte-substratum adhesion (Marin *et al.*, 2005; Brooks and Dunphy, 2005; Gulii *et al.*, 2009), and higher levels decrease hemocyte-substratum adhesion (Marin *et al.*, 2005) but stimulate late immune reactions, such as phagocytosis and possibly nodulation (Baines and Downer, 1992; Stanley and Miller, 2008). A similar bimodal effect of cAMP on mammalian leukocyte adhesion is apparent where lower intracellular cAMP increases granulocyte adhesion to a substratum (Bryant and Sutcliffe, 1974; Derian *et al.*, 1995), and higher levels stimulate cell-cell interactions to induce macrophage granulomatous reactions (Wahl *et al.*, 1979). That some studies report only loss of adhesion to a substratum corresponding with high levels of intracellular cAMP, as seen in insect hemocytes and human neutrophils (Bloemen *et al.*, 1997; Marin *et al.*, 2005), may represent cell densities too low to promote cell-cell adhesion. Other hemocyte adhesion factors include phosphotyrosine kinases and calcium-dependent PKC; these active enzymes decreasing hemocyte adhesion (Zakarian *et al.*, 2003).

Late cellular immune responses

Depending on the type, concentration and size of a microbe, one of the following three responses will occur post-adhesion: phagocytosis, nodulation, or encapsulation. The process of nodulation is typically accompanied by phagocytosis, where phagocytic hemocytes remain capable of participating in nodulation (Horohov and Dunn, 1983; Ratcliffe and Gagen, 1976; Howard *et al.*, 1998). There is also functional heterogeneity within the hemocyte subtypes (Dunphy *et al.*, 1986; Nardi, *et al*. 2006); their functions during these immune processes have not been completely elucidated.

Phagocytosis

The process of phagocytosis occurs in response to low numbers of small microorganisms, such as bacteria and yeasts, in the insect's hemocoel (Horohov and

Dunn, 1983). Along with infecting microbes, insect hemocytes can also phagocytose apoptosed cell debris (Ling and Yu, 2006a). The process of immunocyte phagocytosis is similar across the animal kingdom, including between insects and mammals (Bayne, 1990). After adhesion, the antigen is internalized by being tightly enveloped (zipper mode) or loosely enclosed (macropinocytosis) by membrane-extended pseudopods or sinking in to the surface of the cell (trigger mode; Costa *et al.*, 2005), similarly seen in mammals (Alpuche-Aranda *et al.*, 1994; Swanson and Baer, 1995). The internalized microbe is isolated from the cytosol inside a phagosome which subsequently fuses with lysosomes (Bayne, 1990). Fusion with the lysosome allows degradative enzymes and free radicals to kill the microbe. Mammalian intracellular pathogens, such as *Legionella pneumophila*, can prevent fusion of lysosomes with the phagosome allowing them to safely multiply inside macrophages after phagocytosis (Flannagan *et al.*, 2009), as do entomopathogens, such as the fungus *Metarhizium anisopliae* whose conidia internalized by tick cell lines remain viable, and germinate and grow inside the host cell (Kurtti and Keyhani, 2008).

The hemocytes responsible for phagocytosis of foreign material depend on the insect species. In the lepidopterans *G. mellonella*, *Bom. mori*, *Sp. exigua*, and *Ps. includens*, both granular cells and plasmatocytes are capable of phagocytosis (Ratcliffe and Rowley, 1974; Wago, 1983a; Pech *et al.* 1994; Tojo *et al.*, 2000; Shrestha and Kim, 2007), whereas in *Ma. sexta* plasmatocytes are the primary phagocytes (Ling and Yu, 2006a). In the dipteran, *D. melanogaster*, only the plasmatocytes, which resemble lepidopteran granular cells, are involved in phagocytosis (Meister and Lagueux, 2003). Furthermore, the rate of phagocytosis differs between and within hemocyte types with certain plasmatocyte subtypes displaying extreme phagocytic activity (Tojo *et al.*, 2000; Dean *et al.*, 2004b).

Phagocytosis signal transduction

If hemocytic protein release is inhibited or disturbed, phagocytosis is severely compromised (Foukas *et al.*, 1998; Brooks and Dunphy, 2005), which, if reflecting limited release of adhesion molecules, implies adhesion can occur without internalization but internalization cannot occur without adhesion. To determine the signaling

components of phagocytosis the initial adhesion reaction and subsequent internalization process must be considered separately. Drugs affecting actin polymerization, such as cytochalasin D, will prevent internalization by hemocytes from the medfly, *C. capitata* (Foukas *et al.*, 1998). The actin-based filopodial protusions on *Bom. mori* granular cells elongate after recognition of foreign material, such as glass and interspecies epithelial cells (Wago, 1983b), and inhibiting actin polymerization will prevent their phagocytosis of sheep erythrocytes (Wago, 1984). Hemipteran plasmatocytes display filopodia but their involvement in phagocytosis is not always necessary (Borges *et al.*, 2008). Activation of Src-family kinases, signaling factors influencing actin cytoskeleton reorganization, can stimulate phagocytosis in insect hemocytes (de Winter *et al.*, 2007), as seen in mammalian neutrophils (Hsu *et al.*, 2003). Focal adhesion kinase (FAK), a tyrosine kinase associated with focal adhesions and responsible for generating signals from surface receptors, such as integrins, is involved in phagocytosis and associates with similar molecules in insect and mammalian immunocytes, including Src and extracellular signal-regulated kinase (ERK; Lamprou *et al.*, 2007; Schlaepfer *et al.*, 1999). Other positive effector molecules involved in phagocytosis include phosphoinositide 3-kinases, which influence membrane trafficking and are associated with FAK (de Winter *et al.*, 2007; Schlaepfer *et al*., 1999). The signaling molecules mitogen-activated protein kinases (MAPK), including ERK, c-Jun N-terminal kinase (JNK), p38, and p44, are activated during phagocytsis of *Es. coli* in dipteran hemocytes (Lamprou *et al.*, 2005; Lamprou *et al.* 2007), however, JNK is not involved in phagocytosis of the same foreign material in lepidopteran hemocytes (de Winter *et al.*, 2007), which demonstrates evolutionary differences between insect orders. Rac2, a member of the Ras-superfamily of small GTPases, contributes to phagocytosis of Gram-negative and -positive bacteria in *D. melanogaster* hemocytes (Avet-Rochex *et al*., 2007). In mammalian neutrophils, Rac1 and Rac2, both of which act downstream of membrane activation, contribute to bacterial killing, whereas only Rac2 is involved in the internalization process (Koh *et al.*, 2005). Rac2 can modulate the respiratory burst pathway in mammalian neutrophils (Glogauer *et al.*, 2003), producing reactive oxygen species contributing to the destruction of internalized microorganisms (Babior, 1999). Homologous proteins involved in neutrophil superoxide production have been found in *G. mellonella* hemocytes, microbial killing in

these immunocytes being inhibited by similar NADPH-oxidase inhibitors (Bergin *et al.*, 2005).

Rapid acidification of the phagosome activates hydrolytic enzymes in mammalian blood cells, and this occurs similarly in molluscan hemocytes (Beaven and Paynter, 1999). Hydrolases associated with molluscan hemocyte phagosomes consist of carbohydrases, esterases, phosphatases, and transferases (Moore and Gelder, 1985; Gelder and Moore, 1986), and are similarly seen in leukocyte phagosomes (Haas, 2007). Research on insect hemocyte phagosome hydrolases is lacking. The protein interactome of dipteran hemocyte phagosomes is quite complex involving at least 617 proteins potentially associated with the structure, including 100 mammalian orthologs, with many molecules involved in distinct phagocytic pathways against Gram-positive and/or -negative bacteria (Stuart *et al.*, 2007). The process of phagocytosis shares similar signaling molecules as nodulation; in that eicosanoids are known to stimulate both reactions (Mandato *et al.*, 1997). This may allow both reactions to occur simultaneously depending on the concentration of microbes in a given area in the insect hemocoel. Furthermore, the entomopathogenic bacterium, *Xenorhabdus nematophila*, escapes phagocytosis by *Sp. exigua* hemocytes by inhibiting phospholipase (PL) A_2. Active PKA, a downstream effector molecule of cAMP, suppresses phagocytosis in *G. mellonella* hemocytes, while its specific inhibitors can enhance phagocytosis of *Ba. subtilis* (Brooks and Dunphy, 2005). Pertussis toxin, a $G\alpha_i$ stimulant acting to suppress adenylate cyclase to lower intracellular levels of cAMP, can inhibit fMLP-enhanced hemocyte phagocytosis of latex beads by *Acheta domesticus*, *Zophobas morio*, and *G. mellonella* hemocytes (Garcia-Garcia *et al.*, 2009). In mammals, elevated activity of phosphatases limits the phagocytic ability of macrophages (Zhang *et al.*, 2000), which may counteract the effects of the aforementioned kinases. Phosphatases may play significant roles in insect hemocytes by distinguishing the type of immune response, i.e. phagocytosis or nodulation against small or large numbers of microbes present in the hemocoel, but this remains unclear. Furthermore, phagocytosis is used as a means to remove non-self material from circulation; some hemocyte subtypes from *G. mellonella* appear incapable of phagocytosis and simply remove particulates from the hemocoel by adhering to them (Ehlers *et al.*, 1992). How pathogenic microbes are phagocytosed by hemocytes and

avoid destruction once internalized remains unknown (Anderson *et al.*, 1973; Canesi *et al.*, 2002).

Nodulation and encapsulation

When small foreign particulates, such as bacteria, yeasts, and synthetic microbeads, occur in the hemocoel in numbers too large to be effectively phagocytosed, granular cells and plasmatocytes interact to form multicellular structures called nodules to quanrantine the material (Ratcliffe and Gagen, 1976; Ratcliffe and Gagen, 1977). When hemocytes encounter larger non-self material, such as nematodes, some protozoans, certain stages of fungal development (hyphae and conidiophores), and large synthetic material (agarose and Sephadex beads), a process similar to nodulation occurs, encapsulation, whereby the non-self particulates are contained in a cellular capsule (Schmit and Ratcliffe, 1977). In mammals, biotic and abiotic material difficult to eliminate is encapsulated by way of granulomatous reactions (Majno and Joris, 2004), the resulting structures (granulomas) resemble nodules and capsules in insects. The formation and structure of nodules and capsules in insects resemble also granulomas and capsules from molluscs (Sminia *et al.*, 1974; Harris and Cheng, 1975; Sangster and Smolowitz, 2003) and annelids (Cooper, 1968; Porchet-Henneré *et al.*, 1987). Due to the similarities between nodules and capsules in insects, both in terms of morphology and signal transduction, they will be discussed together.

In *G. mellonella*, nodulation rapidly begins when the granular cells degranulate upon contact with the microbes releasing extracellular matrix proteins and bactericidal enzymes, including phenoloxidase, to form microaggregates composed primarily of granular cells and a few plasmatocytes around the small foreign microorganism (Ratcliffe and Gagen, 1977; Chain and Anderson, 1983; Ling *et al.*, 2005). The microaggregates settle, adhering to surrounding tissues (~5 min) while other granular cells attach to and further expand the melanizing microaggregate. Some plasmatocytes are incorporated also into this microaggregate. Between 2-4 h, plasmatocytes begin to adhere to and spread on the granular cell microaggregates, this reaction continuing 24 h post-infection (Ratcliffe and Gagen, 1977). Nodulation likely ceases as the last plasmatocyte layer is perceived by surrounding hemocytes as normal self, perhaps due to their release of basement

membrane-related proteins (Pech *et al.*, 1995). This process is analogous to the formation of mammalian immune/hypersensitivity granulomas against small microorganisms, wherein the core is composed of tightly packed macrophages having phagocytosed the immune elicitor, followed by a layer of lymphocytes and a final loose layer of fibroblasts (Majno and Joris, 2004).

The process of cellular encapsulation in *G. mellonella* begins when granular cells contact the large microorganism's surface and lyse releasing sticky coagulum (likely ECM proteins), whereby other granular cells can adhere and continue to release ECM proteins (Schmit and Ratcliffe, 1977). Plasmatocytes then adhere to and spread over areas of the foreign material previously contacted by the granular cells. Plasmatocytes form several flattened layers on the microorganism (Schmit and Ratcliffe, 1977). These multicellular masses composed of granular cells and plasmatocytes formed on the microorganism spread laterally over the foreign surface as more hemocytes are incorporated and eventually fuse with masses from other contact sites to completely encapsulate the microorganism (Nardi *et al*, 2006). The capsule settles onto and adheres to the surrounding tissues much like the nodules; encapsulation is terminated when the last plasmatocyte layers do not flatten, retaining a more spherical shape (Schmit and Ratcliffe, 1977). In the lepidopteran, *Ps. includens* the granular cells form a final layer of hemocytes above the unflattened plasmatocytes, ceasing the encapsulation event (Pech and Strand, 1996). Plasmatocytes eventually induce these granular cells to undergo apoptosis (Pech and Strand, 2000). Whether melanization of the capsule occurs depends on the lepidopteran species and type of capsule elicitor; nylon monofilaments (Renault *et al.*, 2002), immulectin-2-conjugated nickel agarose beads (Ling and Yu, 2006b), and the dead nematode *Steinernema carpocapsae* (Walter *et al.*, 2008) induce melanization, whereas xenografted insect nerve cord (Schmit and Ratcliffe, 1977) and live *St. carpocapsae* (Walter *et al.*, 2008) do not. Flavin adenine dinucleotide (FAD) glucose-dehydrogenase is present in granules of *Ma. sexta* plasmatocytes and granular cells and may participate in the phenoloxidase and sclerotization processes during the early and late encapsulation response, respectively (Cox-Foster and Stehr, 1994). Mammalian macrophages produce also immune/hypersensitivity granulomas against large microorganisms, such as multicellular parasites, resembling insect capsules. In another

type of granuloma, called foreign body granuloma, macrophages and giant cells adhere to the surface of large synthetic material, such as surgical sutures and splinters, followed by the addition of a loose coat of fibroblasts to effectively quarantine the non-self material (Majno and Joris, 2004), and resembling also the insect capsule.

Nodulation and encapsulation signal transduction

Eicosanoids are signal transduction molecules that affect both nodulation and encapsulation processes from different insect orders, including lepidopterans (*G. mellonella*, *Ma. sexta*), coleopterans (*Z. atratus*), hemipterans (*Rhodnius prolixus*) and dipterans (*D. melanogaster*; Miller *et al.*, 1994; Mandato *et al.*, 1997; Howard *et al.*, 1998; Carton *et al.*, 2002; Garcia *et al.*, 2004). Eicosanoids, long-chain oxygenated polyunsaturated fatty acids derived from arachidonic acid, give rise to four families of molecules: prostaglandins, leukotrienes, prostacyclins, and thromboxanes (Funk, 2001). Prostaglandins (PGs) and leukotrienes, products of cycloxygenase and lipoxygenase, respectively, have been more intensely studied in insects than the latter two families (Stanley and Miller, 2006). In lepidopterans, the role of cycloxygenases has been elucidated during nodulation of bacteria and carboxylated latex microspheres (Mandato *et al.*, 1997; Howard *et al.*, 1998), whereas lipoxygenases appear to modulate the process against fungi (Lord *et al.*, 2002). Several PGs have been identified in plasma and hemocytes of the lepidopteran *Pseudaletia unipuncta*, including $PGF_{2\alpha}$, PGE_2, PGD_2, and PGA_2, but only levels of $PGF_{2\alpha}$ increase above control levels 30 min post-bacterial infection (Jurenka *et al.*, 1999). However, different levels and ratios of PGs in tissues (muscles and reproductive organs) of different insect orders do occur (Murtaugh and Denlinger, 1982), and may imply a diversity of function in PG type across these orders. The activity levels of PLA_2, responsible for releasing arachidonic acid from membrane phospholipids, are elevated by bacteria as early as 30 sec after infection in *Ma. sexta* (Tunaz *et al.*, 2003). *In vitro* biosynthesis of PGA_2, PGD_2, PGE_2, and $PGF_{2\alpha}$ from microsome-enriched *Ma. sexta* hemocyte lysates occurs as early as 2 min (Gadelhak *et al.*, 1995). Collectively, this advocates the potential role of eicosanoids as early mediators of both the nodulation and encapsulation responses. In human umbilical endothelial cells, PGE_2, $PGF_{1\alpha}$, and $PGF_{2\alpha}$ synthesis occurs as early as 1 h post-stimulation by *Candida*

albicans (Filler *et al.*, 1994), while in the murine fibroblast cell line, Swiss 3T3, PGE_2 synthesis after human platelet-derived growth factor stimulation occurs within 10-15 min (Habenicht *et al.*, 1985). Furthermore, PGE_2 can stimulate $\alpha_v\beta_3$ integrin-dependent adhesion and spreading of endothelial cells onto fibronectin by signaling through the cAMP/PKA pathway (Dormond *et al.*, 2002). Activation of human neutrophil aggregation depends on lower intracellular cAMP levels (Spagnuolo *et* al., 1987), but as to whether this event is PGE_2-dependent is unclear. However, neutrophils do not typically aggregate around an immune elicitor, but do so as a mechanism to help recruit other circulating neutrophils to the site of inflammation (Rochon and Frojmovic, 1992). Macrophages from acute granulomas against *Schistosome mansoni* eggs produce and secrete more prostaglandins than macrophages from acute granulomas against Sephadex beads (Chensue *et al.*, 1983). Mycobacterium infection causes a PGE_2-dependent increase in intracellular cAMP during the initial stages (< 30 h) of macrophage granulomatous reactions (Wahl *et al.*, 1979), these intracellular levels of cAMP later decrease over time (30 – 192 h; Wahl *et al.*, 1979; Bonta *et al.*, 1981). Also, cyclooxygenase, but not lypoxygenase, products are metabolized by macrophages challenged with mycobacterium metabolites, which normally induce granulomas (Wahl *et al.*, 1979) and those undergoing immune/hypersensitivity granulomatous reactions against *Sc. mansoni* (Tripp *et al.*, 1988). This might suggest similar functions of prostaglandins and cAMP in mammalian granuloma formation and insect nodulation and encapsulation. In contrast, eicosanoids derived from lipoxygenase (Capodici *et al.*, 1998), and not cyclooxygenase (O'Flaherty *et al.*, 1979) cause integrin-dependent neutrophil homotypic aggregation, much like nodulation against fungi in *Ma. sexta* (Lord *et al.*, 2002). That PGE_2 influences immunocyte adhesion in insect hemocytes and mammalian macrophages and neutrophils further demonstrates the similarities in signal transduction components between these cell types (Cantarow *et al.*, 1978; Bloemen *et al.*, 1997; Gulii *et al.*, 2009). PG synthesis in insect hemocytes and mammalian cells can show similar kinetics, whole cells capable of producing PGs within 30 min after being stimulated. This time frame corresponds with the early microaggregation event during the nodulation response in insects (Ratcliffe and Gagen, 1977), however, cyclooxygenase-derived products are also known to stimulate plasmatocyte spreading (Mandato *et al.*, 1997;

Kwon *et al.*, 2007), which occurs much later in nodulation. In 3T3 cells, PGE_2 synthesis increases further between 2-6 h, following the initial peak at ~10-15 min, after human platelet-derived growth factor-stimulation (Habenicht *et al.*, 1985). Whether a similar increase in PG production occurs when plasmatocytes begin adhering to microaggregates during nodulation is not clear.

Hemocytes *in vitro* form also microaggregate-like structures (henceforth referred to as *in vitro* microaggregates), which are anchored atop a monolayer of plasmatocytes (Walters, 1970; Miller and Stanley, 2001). Experiments have shown that both *in vivo* and *in vitro* microaggregates are primarily granular cells, having also few incorporated plasmatocytes (Ratcliffe and Gagen, 1977; Davies and Preston, 1985). In *Ma. sexta*, *in vitro* microaggregates induced from bacterial and LPS preparations are inhibited by cyclooxygenase inhibitors, but not by lipoxygenase inhibitors and rescued by the addition of prostaglandins (Miller and Stanley, 2001; Miller and Stanley, 2004), indicating extracellular prostaglandins participate in microaggregate formation. During infection, *G. mellonella* plasmatocytes are the first hemocyte type to exit circulation and adhere to surrounding tissues (Chain and Anderson, 1982). *In vivo* microaggregates collected from the hemolymph of bacteria-injected *Ma. sexta* larvae do not display plasmatocytes rosetting around clusters of granular cells (Stanley and Miller, 2006), as *in vitro* microaggregates, and presumably remain unattached to a substratum. However, time-lapse sequence of *in vitro* microaggregates from larvae of the lepidopteran *Ephestia kuehniella* show plasmatocytes within the coacervate spreading beneath the phase bright hemocyte clump and anchoring the hemocyte microaggregate to the substrata (Davies and Preston, 1985), possibly implying that *in vivo* and *in vitro* hemocyte microaggregates require plasmatocytes to adhere to a substratum. Plasmatocytes are the only hemocyte type capable of migration in insects, including coleopterans (beetles) and lepidopterans (Clark and Harvey, 1965; Baerwald and Boush, 1970; Davies, 1983), a process influenced by the biogenic amine octopamine in *G. mellonella* (Diehl-Jones *et al.*, 1996). In six lepidopteran genera, plasmatocytes, after clumping, migrate away from high hemocyte density areas, such as hemocyte microaggregates, to areas of lower hemocyte density *in vitro* (Walters, 1970; Davies, 1983; Davies and Preston, 1985). The ruffled fan-shaped, leading edge of the plasmatocyte is directed outwards (direction of filopodia)

from the *in vitro* hemocyte microaggregates, suggesting plasmatocyte migration occurs away from the cell coacervate (Walters, 1970). Similar to *in vitro* microaggregates, plasmatocytes incorporated in the *in vivo* microaggregate may anchor the structure to surrounding tissues, while other similarly incorporated plasmatocytes and those settled on nearby tissues are responsible for further anchoring the growing nodule or capsule.

Plasmatocytes, focal points for granular cell attachment, mediate this attachment by a neuroglian/integrin interaction on the surface of these cells (Zhuang *et al.*, 2007a). The majority of neuroglian-negative plasmatocytes and granular cells in *Ma. sexta* form *in vitro* microaggregates, which adhere to focal, neuroglian-positive plasmatocytes (Nardi *et al.*, 2006), demonstrating functionally distinct hemocyte subtypes. Granular cells mediate homotypic and heterotypic cell-cell interactions by releasing extracellular matrix proteins, such as lacunin, to coat adjacent cells, whereby cell-cell adhesion occurs by way of clustering cross-linking integrin receptors (Nardi *et al.*, 2005). Furthermore, hemocyte-hemocyte interactions can occur also between transmembrane receptors (Zhuang *et al.*, 2007b), similarly seen in human granulocyte and lymphocyte homotypic aggregation (Okuyama *et al.*, 1992; Simon *et al.*, 1992; Cao *et al.*, 1997). The inhibition of neuroglian, tetraspanin, and integrin molecules, using monoclonal antibodies and silencing RNA, mediating these cell-cell interactions will prevent encapsulation of foreign material (Wiegand *et al.*, 2000; Nardi *et al.*, 2006; Zhuang *et al.*, 2007a; Zhuang *et al.*, 2007b). This is similar to the multicellular nodulation and encapsulation reactions in insects and crustaceans, wherein the addition of RGD peptides will inhibit these reactions (Pech and Strand, 1995; Pech and Strand 1996; Martin *et al.*, 1998), suggesting the involvement of plasma or membrane-associated RGD-related proteins. Integrins are crucial components of the nodulation and encapsulation responses, as evidenced by increased expression of both α and β integrin subunits in hemocytes from *Ps. includens* during encapsulation (Lavine and Strand, 2003), and the ability of integrin-specific monoclonal antibodies to inhibit encapsulation and hemocyte spreading behaviors in *Ma. sexta* (Wiegand *et al.*, 2000). In mycobacterium-stimulated THP-1 cells, derived from monocytic/macrophage leukaemia cells, cell-surface $\alpha_L\beta_2$ (LFA-1, CD11a/CD18) and $\alpha_M\beta_2$ (CR3, CD11b/CD18) integrin expression is increased above non-stimulated cells (Ramirez *et al.*, 1994), suggesting their involvement in macrophage homotypic adhesion during immune

granuloma formation further implicating the roles of integrins in immunocyte interactions in animals.

The later stages of nodulation and encapsulation include plasmatocyte adhesion to and spreading onto outer granular cell layers of microaggregates (Ratcliffe and Gagen, 1977; Schmit and Ratcliffe, 1977). Several chemokines and intracellular signalling molecules responsible for hemocyte spreading have been elucidated (Gillespie *et al.*, 1997). Lacunin, an intracellularly-stored ECM protein released after non-self recognition and whose expression continues during bacterial infection, is responsible for *in vitro* homotypic and heterotypic hemocyte interactions and possibly *in vivo* nodule and capsule formation (Nardi *et al.*, 2005; Kanost and Nardi, 2010). Members of the lepidopteran ENF peptide family include the hemocyte-released proteins plasmatocyte spreading peptide (PSP), paralytic peptide (PP), and growth blocking peptide and all promote plasmatocyte spreading (Clark *et al.*, 1997; Wang *et al.*, 1999; Strand *et al.*, 2000). PSP from *Ps. includens* is released by granular cells and inhibits also granular cell attachment to substrata (Strand and Clark, 1999). Furthermore, both *Ps. includens* PSP and *Ma. sexta* PP can induce plasmatocyte aggregation (Strand and Clark, 1999; Wang *et al.*, 1999). Another member of the ENF peptide family is hemocyte chemotactic peptide found in *Pse. separata*, which acts as a chemoattractant and promotes aggregation (Nakatogawa *et al.*, 2009). Whether these ENF peptides are involved in compartmentalizing the different phases of nodulation and encapsulation is not clear. There are additional factors not directly produced by hemocytes that affect hemocyte movement. Bacterial fMLP can stimulate chemotactic behaviors in *Gromphadorhina portentosa*, *Ac. domesticus*, *Z. morio*, and *G. mellonella* hemocytes (Garcia-Garcia *et al.*, 2009) and could attract the blood cells to nodules.

Cell-free, humoral immune response

The cell-free, humoral response is characterized by soluble, plasma proteins functioning to eliminate infectious microorganisms. There are two types of humoral responses: innate and induced. Innate humoral responses include the constitutive (and inducible) plasma proteins apolipophorin III, prophenoloxidase, lysozyme, and lectins/agglutinins which can act as pattern recognition proteins and/or antimicrobial

agents and influence the cellular immune responses (Gillespie *et al.*, 1997). The induced humoral response is characterized as the *de novo* synthesis of small, antimicrobial peptides against bacteria and fungi occurring hours after the initial recognition event (Lemaitre and Hoffmann, 2006). The following is a highlight of the most well-defined humoral proteins, and in the case of the induced humoral peptides, their signal transduction components will be reviewed.

Innate humoral response

Apolipophorin III

Apolipophorin III (ALP III), having been introduced earlier as a PRR (see section *Immune recognition*), occurs in the fat body, hemocytes and plasma of non-immune larval *G. mellonella*; its production ceases in the pupal and adult stages (Halwani *et al.*, 2001). ALP III acts to increase antibacterial and lysozyme-like activity during bacterial infection in *G. mellonella* (Wiesner *et al.*, 1997; Niere *et al.*, 1999; Halwani and Dunphy, 1999). ALP III detoxifies LTA from the Gram-positive bacteria *Ba. subtilis, Enterococcus hirae,* and *Streptococcus pyogenes* and LPS from *X. nematophila* negating their effects on *G. mellonella* hemocytes while inhibiting *Ba. subtilis*-induced phenoloxidase activity and preventing *X. nematophila* LPS inhibition of phenoloxidase activiation (Dunphy and Halwani, 1997; Halwani *et al.*, 2000). *G. mellonella* ALP III impairs plasmatocyte adhesion to and possible phagocytosis of the Gram-positive bacteria *Ba. subtilis* (Zakarian *et al.*, 2002), but will opsonize and enhance plasmatocyte phagocytosis of yeast (Wiesner *et al.*, 1997), perhaps indicating functional heterogeneity to different microbial species. Furthermore, *G. mellonella* ALP III will enhance encapsulation of beads and *in vitro* hemocyte microaggregation (Dettloff *et al.*, 2001), but impair plasmatocyte adhesion and spreading on glass surfaces (Zakarian *et al.*, 2002) in keeping with the fact that late ALP III-enhanced encapsulated beads have no apparent layers of plasmatocytes (Whitten *et al.*, 2004). Whether ALP III coordinates the phases of nodulation and encapsulation, much like that proposed for the ENF peptides (see section *Nodulation and encapsulation signal transduction*), is not clear.

The sequence of *G. mellonella* ALP III is similar to that of *Ma. sexta* ALP III (Weise *et al.*, 1998), which shows functional and structural homology to human

apolipoproteins (Cole *et al.*, 1987). Apolipoprotein E can bind and detoxify LPS and also enhance granulocyte phagocytosis of Gram-negative bacteria in mice (Feingold *et al.*, 1995; de Bont *et al.*, 2000); while apolipoprotein E deficient mice are more susceptible to LPS-induced septic shock (Laskowitz *et al.*, 2000). Furthermore, *G. mellonella* ALP III can react with and induce mammalian erythrocytes to agglutinate (Iimura *et al.*, 1998), implying functional similarities with lipoproteins from mammals, including rabbits (Vulpis *et al.*, 1963).

Phenoloxidase

Upon microbial recognition in the insect hemocoel, the phenoloxidase (PO) cascade is activated alongside the cellular immune events. PO exists as a zymogen [pro-phenoloxidase (proPO)] in either the hemolymph, as in *Bom. mori* (Ashida, 1971), or within hemocytes and is released into the plasma during antigenic stimulation, as in *Ma. sexta* (Jiang *et al.*, 1997). Once activated, PO catalyzes the oxidation of phenols into quinones, which polymerize into toxic melanin (Söderhall and Cerenius, 1998), the oxidation products of this reaction causing direct damage to microbes (Zhao *et al.*, 2007). Melanin is capable of binding and agglutinating, and reducing the motility and viability of microbes (Zhao *et al.*, 2007). Hemolymph proPO is also capable of binding the surfaces of *Ma. sexta* granular cells and spherulocytes, but not oenocytoids, eliciting melanization of these hemocytes upon immune challenge with agarose beads (Ling and Yu, 2005). Not only is PO present in oenocytoids and subtypes of prohemocytes in *Bom. mori*, but also in plasmatocytes and subtypes of granular cells, which alludes to its importance in the encapsulation process (Ling *et al.*, 2005). In *Sp. exigua*, release of the PO zymogen from ruptured oenocytoids is mediated by cyclooxygenase-derived eicosanoids (Shrestha and Kim, 2008), and may be a potential source of plasma proPO. Oenocytoids may release their proPO onto granular cells and plasmatocytes during infection, which could later influence melanization of nodules and capsules, as oenocytoids are absent from these multicellular structures (Ratcliffe and Gagen, 1977; Schmitt and Ratcliffe, 1977). During dipteran melanotic encapsulation, wherein parasitoids are ultimately encased in melanin, the crystal cells (very similar in morphology to lepidopteran oenocytoids) rupture and melanize on the surface of

parasitoids (Nappi and Streams, 1969). There is also evidence to support that LPS-cross-linking to dipteran hemocyte surfaces, a necessary step leading to LPS-internalization, is mediated by phenoloxidase (Charalambidis *et al.*, 1996), and may explain reduced crayfish hemocyte phagocytic functions after phenoloxidase RNA silencing (Liu *et al.*, 2007). Interestingly, neither cell-free plasma nor isolated hemocytes can melanize agarose beads (Ling and Yu, 2005), which implies the compartmentalization of the whole reaction in hemocytes and hemolymph.

Lysozyme

Insect lysozyme is a constitutive and inducible cationic antimicrobial peptide present in insect hemolymph (Gillespie *et al.*, 1997) and shows high sequence similarity between lepidopterous insect species and between lepidopterans and chickens (Jollès *et al.*, 1979). Upon bacterial and fungal infection lysozyme is upregulated, acting to hydrolyze the bacterial peptidoglycan tetrasaccharides, composed of β-1,4 *N*-acetylglucosamine and *N*-acetylmuramic acid, into disaccharides whereby disrupting bacterial cell walls and disrupting N-glycosidic bonds linking carbohydrates and proteins in the cell wall of fungi (Chipman *et al.*, 1968; Marquis *et al.*, 1982; Bae and Kim, 2003; Boguś *et al.*, 2007).

Lectins/agglutinins

Responses to large-scale infections are similar throughout the animal kingdom. There exist the plasma and immunocyte-released factors lectins/agglutinins in sponges, insects, molluscs, and mammals that cause foreign particles to aggregate (Gold *et al.*, 1974; Fisher and DiNuzzo, 1991; Koizumi *et al.*, 1999; Ma and Kanost, 2000; Loimaranta *et al.*, 2005; Watanabe *et al.*, 2006; Yu *et al.*, 2006; Gandhe *et al.*, 2007). Depending on the insect order, such as in lepidopterans, microbial agglutination does not always precede nodulation (Gagen and Ratcliffe, 1976), however, *Ma. sexta* hemocytes do release lectins, such as hemolin, noduler, immulectin-4, and a β-1,3-glucan recognition protein, that are capable of agglutinating microorganisms *in vitro* (Ma and Kanost, 2000; Yu and Kanost, 2002; Yu *et al.*, 2006; Gandhe *et al.*, 2007). The immune cells from higher metazoan orders interact with and remove agglutinated bacteria

(Loimaranta *et al.*, 2005), but whether this occurs with amoebocytes from sponges is not certain.

Induced humoral response

Induced humoral immunity involves the synthesis of small antimicrobial peptides (AMP) predominantly from fat body cells, but also hemocytes. The majority of work on induced humoral immunity has been on *D. melanogaster*, and I will briefly review some important aspects of the field. Induced humoral immunity can be further categorized into systemic and local responses, wherein systemic occurs in the hemolymph of the insects and local occurs when microbial pathogens come in contact with external surface epithilia (Ferrandon *et al.*, 1998). The type of microbe species will influence the expression of specific AMPs through the activation of one of two signaling cascades: Gram-positive bacteria and fungi activate the Toll cascade leading to defensin and drosomycin expression, respectively, and Gram-negative bacteria activate the immunodeficiency (Imd) cascade leading to cecropin and attacin expression, although there is some overlap between pathways (Lemaitre and Hoffman, 2006), e.g. cecropin expression can be induced by both Gram-negative and fungal immunogens in the *D. melanogaster* S2 cell line (Samakovlis *et al.*, 1990). Unlike its homologous mammalian counterparts, the Toll-like receptors and the Interleukin-1 receptor, insect Toll is not a pattern recognition protein (PRR), but is activated downstream of spaetzle, its endogenous ligand, by serine proteases, such as persephone and necrotic, and PRRs, such as peptidoglycan recognition proteins (PGRP), gram-negative binding proteins, and β-glucan binding proteins (Lemaitre *et al.*, 1996; Michel *et al.*, 2001; Ligoxygakis *et al.*, 2002; Gobert *et al.*, 2003). The Imd pathway, resembling the mammalian tumour necrosis factor receptor pathway, acts differently from the Toll pathway, whereby membrane-associated PGRPs are activated by Gram-negative bacterial antigens and activate the cytosolic adaptor Imd (Kaneko *et al.*, 2005; Choe *et al.*, 2005). PGRP-LE, a plasma PRR, interacts with Gram-negative bacterial antigens, activating the Imd pathway by interacting with membrane-bound PGRP-LC (Takehana *et al.*, 2002; Takehana *et al.*, 2004). Both activated Toll and Imd pathways transduce signals inside the cell to ultimately translocate transcription factors to the nucleus and initiate AMP expression

(Lemaitre and Hoffman, 2006). Inhibitors of PLA_2 can inhibit the activation of the Imd pathway in *D. melanogaster* larvae homogenates previously treated with LPS (Yajima *et al.*, 2003), however, in the *D. melanogaster* hemocyte mbn-2 cell line neither arachidonic acid nor dexamethasone participated in LPS-dependent cecropin expression (Samakovlis *et al.*, 1992) possibly reflecting the differences in drug concentrations used in both studies. Contrastly, PLA_2 is activated downstream of Toll and Imd proceeding Gram-positive and -negative bacteria stimulation, respectively, in the coleopteran, *Tribolium castaneum* (Shrestha and Kim, 2010). Dexamethasone, a PLA_2 inhibitor, can inhibit the expression of AMPs in the fat body and midgut of the dipteran, *Anopheles albimanus*, this effect being reversed by the addition of arachidonic acid (García Gil de Muñoz *et al.*, 2008). The $G\alpha_s$ subunit of heterotrimeric G proteins are known mediators of the humoral immune response, as they influence the expression of cecropin genes in the mbn-2 cells after stimulation with LPS and β-1,3-glucan (Samakovlis *et al.*, 1992), but how they associate with either Toll or Imd pathway has yet to be elucidated.

There exist also Toll genes in lepidopteran, *Bom. mori* tissues, including the fat body and reproductive tissues, sharing sequence homology with the Toll genes of *D. melanogaster* and the mosquito, *Anopheles gambiae* and whose expression is suppressed by *Es. coli* and its LPS, *Staphylococcus aureus*, and *Beauvaria bassiana* (Imamura and Yamakawa, 2002; Cheng *et al.*, 2008). Downstream effector molecules of $G\alpha_s$ proteins, such as cAMP and PKA, and also PKC and Ca^{2+} can regulate LPS-induced expression of cecropin B expression in *Bom. mori* (Choi *et al.*, 1995; Shimabukuro *et al.*, 1996). The cellular and humoral immune responses work in synchrony, and whether eicosanoids or G proteins allow cross-talking between both immune responses remains to be determined.

THESIS INTRODUCTION

There are three lepidopteran insects prominently used as innate immune models: *Manduca sexta*, *Bombyx mori*, and *Galleria mellonella* (Kemp and Massey, 2007). The latter is an economically important pest insect to beehives and is used in research on host-human pathogen interactions (Vilcinskas, 2010). The immune responses of lepidopteran insects have been extensively characterized and continue to expand into molecular areas (Ratcliffe and Rowley, 1974; Ratcliffe and Gagen, 1976; Kanost and Nardi, 2010).

This study will examine the involvement of $G\alpha_s$ proteins and integrins in the adhesion-based hemocyte immune responses in *G. mellonella*. cAMP, a secondary effector molecule catalyzed by adenylate cyclase, is involved in both hemocyte-microbe adhesion and later immune responses, such as phagocytosis (Baines and Downer, 1992; Marin *et al.*, 2005; Brooks and Dunphy, 2005; Gulii *et al.*, 2009). Eicosanoids are known to be involved in the multicellular immune response, nodulation, wherein hemocyte-released prostaglandins stimulate hemocyte microaggregation in response to bacteria (Miller and Stanley, 2001, 2004). Stanley and Miller (2006, 2008) have proposed that prostaglandins could act through G protein-coupled receptors to increase intracellular levels of cAMP. Peptide fragments of integrin-binding RGD sequences from extracellular matrix proteins inhibit multicellular immune responses (Pech and Strand, 1995), and integrins have been identified as mediators of *in vitro* hemocyte-hemocyte interactions (Nardi *et al.*, 2006, Zhuang *et al.*, 2007b). However, a link between G proteins, integrins, and nodulation has never been elucidated.

The specific objectives of this thesis are as follows:

1. Characterize the effects of a $G\alpha_s$-modulator, whole cholera toxin, and its subunit components on hemocyte immune responses
2. Identify hemocyte integrin molecules with anti-integrin antibodies, and determine the effects of these antibodies on hemocyte function
3. Provide a link between whole cholera toxin induced hemocyte behavior and integrins

CHAPTER 2: The G protein modulator, cholera toxin, alters hemocyte-substratum and hemocyte-hemocyte interactions

PREFACE

Insects are ideal models to study host-human pathogen interactions. The similarities between the cellular (hemocyte) immune processes of insect and mammalian blood cells, including their signal transduction mechanisms, allow human pathogens to infect insects by way of similar virulence mechanisms (Kemp and Massey, 2007). Insect hemocyte- and mammalian leukocyte-microbial adhesion is necessary for quarantining pathogens in multicellular structures, such as nodules and granulomas, respectively (Ratcliffe and Gagen, 1977; Majno and Joris, 2004). Nodulation is a multi-cellular biphasic immune response to isolate large numbers of unicellular microorganisms in insects, where eicosanoid cyclooxygenase-derived prostaglandins released from hemocytes may stimulate levels of intracellular cAMP in neighboring hemocytes via G protein-coupled receptors (Miller and Stanley, 2001; Stanley and Miller, 2006; Stanley and Miller, 2008). I hypothesized that cholera toxin, a $G\alpha_s$ modulator, could induce reactions similar to nodulation *in vitro* and *in vivo*. Herein, I studied the involvement of $G\alpha_s$ proteins in hemocyte-substratum and hemocyte-hemocyte interactions *in vitro* and *in vivo*.

ABSTRACT

Adhesion is a crucial process in arthropod innate cellular immunity participating in the non-self responses of phagocytosis, nodulation, and encapsulation which facilitate the removal of microorganisms and their components from the hemolymph. Lepidopteran hemocyte-microbe adhesion is mediated by the cAMP/PKA pathway, by which decreases in intracellular levels of these molecules resulting from antigen stimulation increases hemocyte adhesion. Herein, I demonstrate the ability of cholera toxin, an AB_5 toxin capable of activating $G\alpha_s$ subunits of heterotrimeric G protein complexes, to induce a bimodal response in the larval blood cells of the lepidopteran insect, *Galleria mellonella*, resulting in hemocyte-substratum and hemocyte-hemocyte adhesion. Hemocytes respond bimodally to cholera toxin, wherein low (1.2 nM) and high (120 nM) levels induce more hemocytes to aggregate forming *in vitro* microaggregates, although 1.2 nM toxin treatment prevents microaggregate-substratum interaction. I provide evidence that the effects of higher concentrations of toxin are achieved by its B-subunit, whereas those of lower concentrations of toxin are potentially mediated by its A-subunit. *In vitro* hemocyte microaggregates adhered to a monolayer of plasmatocytes arranged in a rosetting pattern around the microaggregate. The formation of *in vitro* hemocyte microaggregates was inhibited by RGD peptides implying the involvement of integrins, as seen in other lepidopteran hemocytes. Injections of cholera toxin resulted in hemocyte mobilization and enhanced bacterial removal from the hemocoel; its B-subunit was responsible for increased bacterial removal at higher concentrations of whole toxin. Cholera toxin also induced nodule-like structures *in vivo* showing some similarities to bacteria-induced nodules, i.e. displaying melanization and adhering to surrounding tissues.

INTRODUCTION

Hemocyte-microbe adhesion is a crucial step in the removal of microorganisms from the insect hemocoel. The process of hemocyte adhesion in inverterbrates, including arthropods (Dunphy and Nolan, 1980; Lackie, 1983; Strand and Lavine, 2001) and molluscs (Wootton *et al*., 2006), can be activated by the physicochemical interactions of non-self materials with hemocyte surface receptors, which is important since hemocyte recognition and adhesion may depend on plasma- or hemocyte-derive enzymes to modify the surface properties of an antigen (Dunphy and Webster, 1991; Ford and Ashton-Alcox, 1998). Microbial recognition is mediated also by cell-free plasma proteins capable of binding to lipopolysaccharides, lipoteichoic acids, and β-1,3-glucans, and include the proteins apolipophorin III, lysozyme, hemolin, noduler, and immunlectin-2 (Koizumi *et al.*, 1999; Yu and Kanost, 2002; Whitten *et al.*, 2004; Gandhe *et al.*, 2007; Lee, 2007). In the hemocoel, the surface charge of the basement membrane can alter hemocyte reactions either by detering recognition of self to cease an encapsulation response or stimulating hemocyte activation during ecdysis (molting)-related tissue remodeling (Wigglesworth, 1956; Rizki and Rizki, 1974; Pech *et al.*, 1995; Pech and Strand; 1995), the latter activation representing the ability of hemocytes to distinguish between abnormal-self and normal-self antigens (Medzhitov and Janeway, 2002). A receptor capable of detecting physicochemical signatures, such as electrostatic charge or hydrophobicity, could bind several types of ligands, which may be the case for the scavenger receptor found on larval *Spodoptera littoralis* hemocytes that binds both lipopolysaccharide and lipoteichoic acid from *Escherichia coli* and *Bacillus subtilis*, respectively (Costa *et al.*, 2005). Moreover, receptors recognizing specific antigen epitopes could also stimulate adhesion (Ohta *et al.*, 2006). The adhesion-based hemocyte immune responses, occurring within minutes (Gagen and Ratcliffe, 1976; Giannoulis *et al.*, 2005), can vary in intensity, depending on charge (Dunphy and Nolan, 1980; Lackie, 1983) or the abiotic or biotic nature of the foreign surface (Scapigliati *et al.*, 1997; Lamprou *et al.*, 2005), and may be due to both epitope and non-epitope, promiscuous surface/scavenger receptors (Ofek *et al.*, 1995; Costa *et al.*, 2005; Ohta *et al.*, 2006). The adhesion event occurs when intra-hemocytic stores of extracellular matrix proteins are released and adhere to surfaces of foreign

material (Ball *et al.*, 1987; Johansson and Söderhall, 1988; Liang *et al.*, 1992; Scapigliati *et al.*, 1997; Holmblad and Söderhall, 1999) allowing RGD-dependent (Pech *et al.*, 1995) and RGD-independent (Lamprou *et al.*, 2005) hemocyte adhesion. The use of glass material as an indication of hemocyte activation/adhesion has been widely used in insect studies (Walters, 1970; Dean *et al.*, 2004a; Giannoulis *et al.*, 2005) as it induces reactions similar to cellular immune responses, such as encapsulation (Brooks and Dunphy, 2005).

Two types of hemocytes participate in adhesion-based cellular immune responses of lepidopterans and larval *Galleria mellonella* in particular: the spherical, phase bright granular cells and amoeboid, phase dark plasmatocytes (Price and Ratcliffe, 1974; Ratcliffe and Gagen, 1977). Hemocytes are capable of isolating unicellular microorganisms, such as bacteria and yeasts, from the hemolymph via two main cellular responses, phagocytosis and nodulation (Gillespie *et al.*, 1997), which act concurrently to remove microbes (Horohov and Dunn, 1983). Individual microbes can be taken up into single hemocytes by means of phagocytosis involving both cell types (Tojo *et al.*, 2000), while huge numbers of microbes are more effectively removed via nodulation which involves interacting granular cells and plasmatocytes (Ratcliffe and Gagen, 1977). During nodulation, the granular cells contact large numbers of bacteria and degranulate releasing sticky coagulum, bactericidal enzymes (Chain and Anderson, 1983), including phenoloxidase (Ling *et al.*, 2005), entrapping the microbes, whilst the slowly melanizing microaggregates settle on and rapidly adhere to surrounding tissues within minutes (Ratcliffe and Gagen, 1976; Ratcliffe and Gagen, 1977). Plasmatocytes subsequently adhere to and spread on the melanized mass of granular cells forming multiple layers around the microaggregate within hours culminating in nodule formation. The intensity of nodulation depends on the concentration of microbes in the hemocoel and the microbial species (Ratcliffe and Gagen, 1976; Howard *et al.*, 1998). *In vitro*, hemocytes form microaggregates comparable to the preliminary stages of nodulation sharing similar signaling mechanisms to the *in vivo* process (Miller and Stanley, 2001; Miller and Stanley, 2004; Stanley and Miller, 2006).

Nodulation involves both hemocyte-antigen and hemocyte-hemocyte interactions. Most work done on elucidating the types of interactions between granular cells and plasmatocytes during cellular immune responses have been for encapsulation.

Encapsulation is the third type of cellular immune response and targets large microorganisms, such as parasitic wasp eggs and nematodes, and is very similar to the nodulation process and its signal transduction (Schmit and Ratcliffe, 1977; Pech and Strand, 1996; Carton *et al.*, 2002; Garcia *et al.*, 2004). RGD peptides, amino acids motifs found in extracellular matrix proteins and targeted by integrins (Hynes, 1992), can inhibit plasmatocyte binding to the early granular cell capsule (Pech and Strand, 1996). Integrin-binding extracellular matrix proteins, such as lacunin, are stored inside granular cells and released during immune challenge to coat other hemocytes and stimulate hemocyte-hemocyte interactions (Nardi *et al.*, 2005), while other proteins, such as tetraspannin, are transmembrane proteins that stimulate also hemocyte aggregation (Zhuang *et al.*, 2007b). Noduler, a small peptide with a reeler domain commonly found in vertebrate extracellular matrix proteins, can bind microbial surface antigens and stimulate the insectan nodulation response, which signifies its importance during the initial, granular cell-directed phase of nodulation (Gandhe *et al.*, 2007).

There exists separate signal transduction molecules mediating the initial hemocyte adhesion response and the subsequent cellular immune responses, such as nodulation. Intracellular signal transduction molecules, such as cyclic adenosine monophosphate (cAMP) and its downstream effector molecule, protein kinase (PK) A, can mediate adhesion; where increased levels of both molecules impair hemocyte-substratum adhesion and removal of bacteria (Marin *et al.*, 2005; Brooks and Dunphy, 2005; Gulii *et al.*, 2009) and active PKA inhibits protein release from hemocytes (Brooks and Dunphy, 2005), while low levels of cAMP stimulate hemocyte-substratum adhesion (Marin *et al.*, 2005; Gulii *et al.*, 2009). Eicosanoids, oxygenated metabolites of arachidonic acid and other polyunsaturated fatty acids, are known to influence both hemocyte-substratum and hemocyte-hemocyte interactions in numerous insect orders and species (Stanley and Miller, 2006; Gulii *et al.*, 2009). In *G. mellonella*, inhibitors of both arachidonic acid release (from phospholipids) and cyclooxygenase inhibit plasmatocyte spreading (Mandato *et al.*, 1997), whereas the addition of arachidonic acid and prostaglandin E_2 can increase *G. mellonella* granular cell and plasmatocyte adhesion (Marin *et al.*, 2005; Gulii *et al.*, 2009). Eicosanoid inhibitors can also inhibit nodulation of latex microspheres and bacteria (Mandato *et al.*, 1997; Howard *et al.*, 1998). The entomopathogenic bacterium,

Xenorhabdus nematophila, prevents the release of arachidonic acid from phospholipids in hemocytes by inhibiting the activity of phospholipase A_2, which ultimately prevents a nodulation response (Shrestha and Kim, 2007). Furthermore, cyclooxygenase inhibitors inhibit lipopolysaccharide-induced *in vitro* microaggregates in *Manduca sexta*, whereas neither lipoxygenase products nor inhibitors have any effect suggesting only prostaglandins are involved in nodulation against bacteria (Miller and Stanley, 2004), but may be involved in processes against fungi (Lord *et al.*, 2002). Prostaglandins released from hemocytes into the hemolymph could potentially stimulate intracellular cAMP via G protein coupled receptors on other hemocytes (Miller and Stanley, 2001; Stanley and Miller, 2008).

Herein, I use whole cholera toxin, an AB_5 toxin capable of activating adenylate cyclase via $G\alpha_s$ subunits of heterotrimeric G proteins (Chinnapen *et al.*, 2007), and its component B-subunit (CTB), and examine their effects on *G. mellonella* hemocytes *in vitro* and *in vivo*.

MATERIALS AND METHODS

Insect culture

Galleria mellonella larvae were raised in 4.5 L jars at an ambient temperature of 28 °C under constant light conditions on a multigrain diet supplemented with glycerol, vitamins, and brood comb (Dutky *et al.*, 1962). Brood comb was generously supplied by Claude Vinet (Miel Claude Vinet, Vaudreuil-Dorion, Quebec). Fifth instar, *ad libitum* larvae weighing 250 ± 10 mg were used. All experiments with the hemocytes, *in vitro* and *in vivo*, were conducted at 37 °C to mimic both temperature conditions in honey bee colonies (Southwick and Heldmaier, 1987) and in artificially reared *G. mellonella* colonies (Buchmann and Spangler, 1991). *G. mellonella* stocks were genetically enhanced by breeding in feral insects collected from apiaries. These feral insects, reared on brood comb only, were made disease-free (normal hemogram) by breeding new generations before their addition to our insect stocks.

Bacterial culture

Stock cultures of the non-pathogenic, Gram-positive bacteria *Bacillus subtilis* (Boreal Biological, Mississauga, ON, Canada) were grown on Luria agar (1 g NaCl, 1 g yeast extract, 2 g bactotryptone, 3 g agar; 200 ml water) at 25 °C and subcultured every fortnight.

For experimental purposes, *Ba. subtilis* was added to 5 ml Luria broth in 20 ml scintillation vials and incubated overnight on a horizontal gyratory shaker (25 °C, 200 rpm). One hundred µl of the bacterial suspension was added to 5 ml Luria broth in a new 20 ml scintillation vial and incubated for 2 h as previously described. Five ml of culture was added to flasks (500 ml) containing 200 ml Luria broth and bacteria were grown to midlog phase (Optical Density$_{660nm}$ = 0.75 A). Bacteria were pelleted (12,000xg, 15 min, 25°C) and resuspended in 10 ml of phosphate-buffered saline (138 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2.8 mM KH_2PO_4, adjusted to pH 6.5 with 6N HCl; PBS) before being killed by ultraviolet light exposure (3 h). Bacteria suspensions (30 µl) were plated onto

Luria agar and incubated at 25℃ and 30℃ (72 h) to ensure complete bacterial death. Dead bacteria provided hemocyte antigen-stimulation devoid of bacterial metabolic activity (Alavo and Dunphy, 2004). Bacteria were subsequently centrifuge-washed (12,000xg, 15 min, 25 ℃) three times in PBS (10 ml) and stored at -20 ℃.

Treatment solutions

Physiological levels of whole, inactivated cholera toxin (CTX), B-subunit of cholera toxin (CTB), and A-subunit of cholera toxin (CTA; BioMol International) were used (Li *et al.*, 2009). RGDS (GenScript) was used to inhibit CTX-induced *in vitro* microaggregate formation, and RGES (Sigma) was used as a control peptide. To diminish non-specific reactions, solutions containing CTX or its components were made prior to the beginning of the experiments to minimize potential reactions to the surfaces of both microcentrifuge tubes (manufacturer's instructions) and microorganisms (McCardell *et al.*, 1987).

Hemocyte adhesion to, aggregation on, and detachment from glass slides *in vitro*

Hemocyte suspensions were made by collecting hemolymph (15 µl) from the third prothoracic proleg of six larvae (chilled on ice; 10 min) into chilled (4 ℃) PBS (1.33 ml). For all experiments, all instruments coming in contact with the hemocytes were chilled (4 °C). Microcentrifuge tubes were gently inverted twice after hemolymph addition to disperse hemocytes and plasma factors preventing hemocyte adhesion to the tube surface and agglutination. Fifty µl of hemocyte suspension containing ~1.2 x 10^5 cells was added to areas (145 mm^2) on new, endotoxin-free slides containing a treatment solution (CTX, CTB, or CTA with or without RGDS or RGES) or control buffer (50 µl). Adhesion assays were performed with hemocytes in diluted hemolymph since plasma is known to affect adhesion (Koizumi et al., 1999; Zakarian et al., 2003). Slides were shaken on a horizontal gyratory shaker (50 rpm) for 30 min [the optimum control reaction time for adhesion (Zakarian *et al.*, 2003)], unless otherwise specified, at 37 °C and ~95% relative humidity (RH). Since *G. mellonella* hemocytes are mononuclear it was possible

to determine the number of hemocytes per *in vitro* microaggregate with the nuclear stain DAPI (Price and Ratcliffe, 1974). Nuclei of aggregated hemocytes detected by fluorescent microscopy and the number of hemocytes per microaggregate under phase contrast microscopy exhibited a 1:1 ratio since their frequencies were not significantly different ($p > 0.05$; Average cells/microaggregate under: Phase contrast microscopy; 100.8 ± 8.4. Fluorescence microscopy; 105.6 ± 8.8; n = 119). Herein, the number of hemocytes in an *in vitro* microaggregate was determined with phase contrast microscopy.

Following hemocyte attachment to the glass slide, several different protocols were followed: In protocol one, to determine the number of individually attached and aggregated hemocytes on the glass after CTX, CTB, or CTA treatment, slides were rinsed twice with PBS (2 ml) and cells fixed in gluteraldehyde vapor for 30 min. Slides were rinsed twice in PBS (2 ml), mounted in 30% glycerol (v/v PBS) and the total number of individually attached hemocytes, total aggregated hemocytes (all cells in all microaggregates in the field) and individual differential cell types were determined (cells/mm^2) with phase contrast microscopy (20X magnification).

In protocol two, to test if CTX caused hemocyte-substratum detachment, cells previously incubated with PBS (50 µl) were rinsed twice with PBS (2 ml), treatment solution (100 µl) added, and the cells incubated (30 min at 37 °C, ~95% RH). Cells were rinsed twice with PBS (2 ml) and fixed in gluteraldehyde vapor for 30 mins, rinsed again with PBS, and mounted in 30% glycerol (v/v PBS). The total individually attached cells and their type (cells/mm^2) were determined with phase contrast microscopy (20X magnification).

In protocol three, to test for hemocyte lysis or reduced adhesion (detected in protocol one) at 1.2 nM CTX, I determined the number of non-attached hemocytes after CTX treatment. Without rinsing the slides, non-attached hemocytes were resuspended with gentle pipetting, collected (~100 µl), added to areas on new slides, and centrifuged on a tissue culture plate rotor (500xg, 5 mins) to immobilize them on the slide. Hemocytes were fixed in 4% formaldehyde and mounted in 30% glycerol (v/v PBS) and the total number of cells/mm^2, individual and aggregated, determined as previously described. For this protocol, the non-attached hemocytes (cells/mm^2) recorded for the control buffer (PBS) were subtracted from values obtained from treatment values (1.2 nM

CTX) because there may exist sub-populations of *G. mellonella* hemocytes, as alluded to for *Ma. sexta* hemocytes (Nardi, *et al.* 2006), which do not adhere to glass.

In protocol four, to test the effects of RGDS on CTX-induced *in vitro* hemocyte microaggregates, hemocytes were concomitantly treated with CTX and RGDS. Following treatment, hemocytes were immediately fixed in gluteraldehyde vapor for 30 min. Since soluble RGD can inhibit hemocyte attachment and spreading (Pech and Strand, 1995), hemocytes on slides could not be rinsed or compressed with a coverslip and cells per microaggregate could not be accurately determined. Thus, a coverslip supported 0.7 mm above the reaction area was applied, similar to a hanging drop assay, and total aggregated area (cumulative area of all microaggregates in a field) and microaggregate frequency was determined under 10X magnification with phase contrast microscopy and quantified with software (Adobe Photoshop CS3). The height of the supported coverslip (0.7 mm) provided a small working distance for microscopy (for a better focus on all hemocytes on the slide, including non-attached cells). To achieve this small working distance and prevent the reaction from spilling over the edges of the reaction area after applying the coverslip 20 µl of treatment-hemolymph mixture was removed from the edges of the reaction area, which removed a negligible number of cells (<0.03% of total cells added to the slide). Lysis alters hemocyte appearance allowing easy detection of lytic cells with phase contrast microscopy and their exclusion from counts. Where applicable, *in vitro* microaggregates (including rosetting plasmatocytes) were defined as containing no less than five hemocytes. A minimum of four fields per slide area were counted per replicate and each experiment contained a minimum of four replicates. Hemocyte types were identified according to Price and Ratcliffe (1974) with phase contrast microscopy.

Mobilization of hemocytes in the insect hemocoel and their adhesion to glass

Insects (chilled on ice; 10 min) were injected with 10 µl of treatment solution (or control buffer) with or without *Ba. subtilis* (6×10^7 cells) at the third prothoracic proleg [total volume inside insect after injection = 60 µl (Gagen and Ratcliffe, 1976)]. Unless

otherwise specified, insects were incubated for 20 min, at 37 °C. After the incubation time, two different protocols were followed.

For protocol one, to test the effects of CTX, CTB, and CTA on hemocyte mobilization in the insect hemocoel or on bacterial removal from the hemocoel, hemolymph (10 µl) was removed from the insect and the concentration of circulating hemocytes or bacteria determined on a hemocytometer.

For protocol two, to test the adhesive properties of the mobilized/circulating hemocytes from insects injected with CTX, CTB, or CTA, hemolymph (10 µl) was removed from the insects and added to areas (145 mm^2) on slides containing PBS (90 µl). Slides were shaken on a horizontal gyratory shaker (50 rpm, 30 min at 37 °C, ~95% RH). The total cell number and type attached to glass were determined with phase contrast microscopy (20X magnification). The data collected from protocol two was standardized by making a ratio of absolute circulating hemocytes found in protocol one to absolute adhered hemocytes found in protocol two in order to compare the adhesive properties of hemocytes between injected treatment concentrations and treatment solutions (i.e. CTX, CTB, and CTA). No microaggregates were observed on the slides, suggesting either no attachment of aggregates to glass or they were not removed from the hemocoel. Where applicable, four fields per slide area were counted for every replicate and each insect was itself a replicate receiving individually prepared treatments with a minimum of 5 replicates per experiment.

Nodule production *in vivo*

Chilled insects (10 min on ice) were swabbed with 70% (v/v) alcohol at the site of injection. Ten µl treatment solution (CTX) or control buffer was injected at the third prothoracic proleg and the larvae incubated for 24 h at 37 °C on regular diet. Insects were monitored for normal behavior and mortality in order to preclude pharmacological effects on hemocyte behavior. Larvae were chilled on ice for 10 min. The *in vivo* hemocyte reaction was arrested by injecting the insect with 4% formaldehyde (50 µl; v/v in PBS) with subsequent incubation on ice for 10 min. Insects were bisected ventrally with mini-scissors and the frequency of nodules determined with a stereo dissecting microscope

(25X magnification). Nodules appeared as conglomerates comprised of a large central nodule and smaller peripheral nodules and were recorded as individual structures. This type of analysis excluded non-melanized microaggregates. Each insect was itself a replicate receiving individually prepared treatments with a minimum of 20 replicates per experiment.

Statistics

All data were analyzed using the 95% confidence limit overlap protocol (Sokal and Rohlf, 1969). Percentage data are recorded as the decoded mean with 95% confidence limits in 2 arcsin $\sqrt{p}$-transformation. Graphic and tabular data are presented as the mean ± standard error of the mean. An *a priori* α value of 0.05 was chosen.

RESULTS

Adhesion to, aggregation on, and detachment from glass slides *in vitro*

In order to examine the effect of CTX on hemocyte attachment to glass, the effective incubation duration of hemocytes with 0, 6, and 60 nM CTX was determined *in vitro*. Total individual attached and aggregated hemocytes were determined after 20 and 30 min incubation, 30 min being the time for maximum adhesion to slides without CTX (Zakarian *et al.*, 2003; Fig. 2.1). Levels of individually attached hemocytes were significantly less ($p < 0.05$) at 30 min incubation than at 20 min for the PBS controls (Fig. 2.1). Levels of total aggregated hemocytes also differed between the incubation times for the PBS controls, aggregation being greater at 30 min. The levels of total aggregated hemocytes increased with increasing CTX concentration after 30 min incubation, whereas the levels marginally decreased with increasing CTX concentration after 20 min incubation. Total individually attached hemocytes decreased in a CTX concentration-dependent manner at 30 min, the effects being less pronounced at 20 min. Because effects were more pronounced at 30 min incubation all subsequent *in vitro* reactions were incubated for this length of time.

The concentration of individually attached and aggregated hemocytes was determined with increasing CTX levels (ranging from 1.2 to 120 nM). In order to compare the effects of CTB and CTA to the effects of CTX, hemocytes were treated with levels of CTB (0, 6, 30, 60, 150, 300, 600 nM) and CTA (0, 1.2, 6, 12, 30, 60, and 120 nM), which, because whole cholera toxin is an AB_5 toxin, corresponded to CTX levels of 0, 1.2, 6, 12, 30, 60, and 120 nM. The number of individually attached hemocytes exposed to 1.2 nM CTX significantly dropped (66%; 39.2-43.3; $p < 0.05$) from the PBS control, at 6 nM adhesion significantly increased (24%; 13.3-13.9; $p < 0.05$) above 1.2 nM levels, and subsequently decreased linearly with increasing CTX concentration to a maximum decline of 27% (15.2-15.9) from the PBS control at 120 nM CTX (Fig. 2.2A). Granular cell and plasmatocyte adhesion followed a similar pattern throughout all the concentrations of CTX, however, plasmatocyte adhesion remained lower at higher concentrations of CTX (6, 12, 30, 60, 120 nM) than did granular cell adhesion. In

contrast, total aggregated cells increased above the PBS control values at 1.2 nM CTX, followed by a significant decrease ($p < 0.05$) at 6 nM (Fig. 2.2A). Higher concentrations of CTX further increased total aggregated hemocytes from control levels nearing a plateau at 120 nM. Adhered *in vitro* microaggregates were phase bright and presented plasmatocytes rosetting around the edges of the microaggregate (Fig. 2.2B). The leading, fan-shaped edges of the plasmatocytes were directed away from the granular cell cluster suggesting exomigration of the plasmatocytes. The sum of total adhering, individual and aggregated hemocytes was statistically the same ($p > 0.05$) with increasing CTX treatment except for a decline at 1.2 nM CTX (Fig. 2.2C), which may reflect reduced adhesion, hemocyte detachment or lysis at this concentration. Preformed monolayers treated with different amounts of CTX, including 1.2 nM, did not show differences ($p > 0.05$) in either total individual or differential hemocyte counts (Table 2.1) implying the drop in total hemocytes at 1.2 nM was not due to hemocyte detachment or lysis. Non-attached hemocytes (Fig. 2.2C) collected from monolayers treated with 1.2 nM CTX were predominantly aggregated hemocytes (individual: 0 cells/mm^2; aggregated: 93.5 ± 2.7 cells/mm^2). The number of non-attached and adhering cells determined from slide areas with hemocytes treated with 1.2 nM CTX were combined, and the resulting total sum was not significantly different from the sum of adhering (individual and aggregated) hemocytes in the PBS control group, or the higher concentrations of CTX, implying loss of adhesion at 1.2 nM (Fig. 2.2C).

To determine if the CTX effect might reflect the influence of its individual components, hemocytes were treated with corresponding levels of CTB or CTA. Total individually attached hemocytes from 0 – 30 nM CTB represented a plateau, whereas those treated with higher concentrations (60 – 600 nM) of CTB decreased linearly ($R = -0.94$, $p < 0.05$; Fig. 2.2D). Both attached granular cells ($R = -0.89$; $p < 0.05$) and plasmatocytes ($R = -0.75$; $p > 0.05$) followed similar patterns and degrees of decreasing attachment levels with increasing CTB concentration. Total aggregated hemocytes increased to 30 nM, declined but not significantly ($p > 0.05$) at 60 nM CTB, and increased to a maximum plateau by 150 nM CTB (Fig. 2.2D). This implied that individual B-subunits can enter cells, similar to B-subunits from the cholera toxin-related heat-labile *Es. coli* enterotoxin (Sixma *et al.*, 1991). Combining both total individually

Table 2.1 Effect of whole cholera toxin on detachment of pre-formed hemocyte monolayers

	Attached hemocytes (cells/mm^2)		
Whole cholera toxin (nM)	Total hemocytes[†]	Granular cells[†]	Plasmatocytes[†]
0.0	396.7 ± 8.9[a]	211.5 ± 6.5[b]	185.2 ± 6.1[c]
1.2	398.8 ± 8.9[a]	202.8 ± 6.4[b]	195.9 ± 6.2[c]
6.0	412.8 ± 9.0[a]	203.1 ± 6.4[b]	209.4 ± 6.5[c]
12.0	400.7 ± 8.9[a]	204.6 ± 6.4[b]	195.6 ± 6.2[c]
30.0	412.8 ± 9.0[a]	201.6 ± 6.3[b]	211.0 ± 6.5[c]
60.0	369.1 ± 8.6[a]	199.9 ± 6.3[b]	169.3 ± 5.8[c]
120.0	419.5 ± 9.2[a]	223.8 ± 6.6[b]	195.2 ± 6.2[c]

[†] Mean ± standard error of the mean, n≥4. Values within a column with the same superscript were not significantly different, $p > 0.05$.

attached and total aggregated hemocytes yielded similar levels of hemocytes between all CTB treatments, implying the absence of lysis. CTA had no effect on total individually attached or total aggregated hemocytes with increasing concentrations (Fig. 2.2E). The inability of CTA to affect hemocyte activity suggests that it is incapable of entering the cells, however, this does not invalidate its involvement in CTX's effect on the hemocytes.

CTX at high concentrations produced total hemocyte counts statistically similar to CTB ($p > 0.05$) indicating CTB may explain higher CTX results. In terms of granular cells, CTB did not change counts until 300 nM after which the concentration of granular cells declined, and CTX effects from 12 – 120 nM were similar to CTB (60 – 600 nM). Plasmatocyte adhesion at levels exceeding the lowest concentration of CTX (1.2 nM) and CTB (6 nM) were identical ($p > 0.05$). Collectively the data supports an immunological role for hemocytes treated with high concentrations of CTB. CTX elevated the total number of cells that formed *in vitro* microaggregates, but CTB had no effect until 30 nM, elevating total aggregated hemocytes to the CTX values (6 – 120 nM).

Inhibition of *in vitro* hemocyte microaggregate formation by RGD

Since RGD peptides immobilized to Sephadex beads facilitate *Pseudoplusia includens* hemocyte adhesion promoting encapsulation and soluble RGD peptides inhibit this reaction (Pech and Strand, 1995), RGDS tetrapeptides were similarly used to inhibit CTX-induced *in vitro* microaggretaion. CTX concentrations of 1.2 and 120 nM were chosen due to CTX's bimodal increase of *in vitro* microaggregations at these treatment concentrations (see Fig. 2.2A). Five mM soluble RGDS peptide significantly inhibited the total aggregated hemocyte area by 50% (27.5-32.1; $p < 0.05$) and microaggregate frequency by 50% (17.0-27.4; $p < 0.05$) at both 1.2 and 120 nM CTX compared to the absence of the tetrapeptide, whereas RGES had no effect (Fig. 2.3A, B). CTX (1.2 nM)-treated hemocytes incubated with RGDS (5 mM) show less microaggreation than without RGDS or with RGES (5 mM) (Fig. 2.3C,D,E). Effects of RGDS on hemocytes treated with 1.2 nM or 120 nM CTX were the same ($p > 0.05$). Hemocytes displayed similar levels of total aggregated area and microaggregate frequency at both concentrations of CTX without RGDS (Fig. 2.3A) suggesting that 1.2 and 120 nM CTX have the same

effect on hemocyte microaggregation magnitude except that *in vitro* microaggregates formed at 1.2 nM CTX do not adhere to glass (see Fig. 2.2A,C). RGDS did not lyse or detach hemocytes from preformed monolayers after 30 min incubation (total hemocyte counts from PBS control: 412 ± 9.1 cells/mm^2; 5mM RGDS: 401 ± 8.9 cells/mm^2), thus the more uniform distribution of the hemocytes treated with RGDS compared to the samples with RGES and the buffer control represents inhibition of *in vitro* microaggregate formation.

Effect of CTX and its components the A- and B-subunits on the concentration of circulating hemocytes in the larval hemocoel and the ability of these hemocytes to adhere to glass

Hemocytes withdraw from or enter (a form of hemocyte mobilization) the circulation by respectively adhering or losing affinity to surrounding tissues, so modulating hemocyte adhesion can alter the number of cells in circulation (Wigglesworth, 1956; Jones, 1962; Gagen and Ratcliffe, 1976). Since reaction times are shorter *in vivo* than *in vitro* due to differences in plasma factors and their concentrations (Ehlers *et al.*, 1992), incubation duration for maximum CTX effect on total hemocyte counts was determined *in vivo* using similar times as *in vitro* (see Fig. 2.1). Twenty minute incubation produced the most pronounced effect on the concentration of circulating hemocytes (Fig. 2.4). Increasing circulating cells in the PBS control with increasing time is typical of a wound response inflicted by the syringe (Cherbas, 1973; Halwani *et al.*, 2000). However, with increasing incubation the difference between circulating cells in insects injected with PBS and 6 and 60 nM CTX decreased over time suggesting either re-attachment of CTX-sensitive hemocytes to surrounding tissues or toxin removal from or toxin inactivation in the hemolymph. Herein, treatment concentrations used refers to final concentrations inside the insect. Because the effects of CTX were more pronounced at twenty minutes, this time was chosen for subsequent *in vivo* experiments.

The concentration of circulating hemocytes after 20 minuntes was determined with increasing CTX (ranging from 1.2 to 120 nM), CTB (ranging from 6 to 600 nM), and CTA (ranging from 1.2 to 120 nM). Levels of circulating hemocytes peaked at 6 nM

CTX, followed by a drop at 12 nM and rose to a plateau by 60 nM, with hemocyte levels similar to those at 6 nM (Fig. 2.5). CTB caused an initial decrease in circulating hemocytes plateauing from 6 nM to 150 nM, followed thereafter to a maximum increase that plateaued starting at 300 nM, the latter two effects being similar to the corresponding concentrations of CTX (Fig. 2.5). Molar equivalents of CTA had no effect on the concentration of circulating hemocytes implying the inability of CTA to penetrate the hemocytes. The effect of CTX on circulating hemocytes was opposite the pattern seen *in vitro* (see Fig. 2.2A), albeit the reaction taking place at higher levels of CTX suggesting a tissue dilution effect *in vivo*.

For the following experiments, concentrations of 6, 12, and 60 nM CTX (corresponding to 30, 60, and 300 nM CTB), were chosen since 6 and 60 nM represent the most pronounced effects of CTX *in vivo* (see Fig. 2.5), while 12 nM represents an intermediate concentration between the bimodal effects of 6 and 60 nM. Glass slides were used to determine the adhesive properties of the circulating hemocytes remaining in the larval hemocoel after CTX, CTB, or CTA injections. Ten μl of hemolymph was removed and added to the slides. The total number of adhering hemocytes decreased from 6 nM CTX-injected insects compared with PBS-injected insects, whereas adhesion levels increased from insects injected with 12 and 60 nM CTX (Fig. 2.6A). Levels of attached granular cells and attached plasmatocytes followed the same pattern in insects injected with 0, 6, and 12 nM CTX, however, adhering granular cell levels linearly increased with CTX concentration from 6 – 60 nM, while adhering plasmatocytes plateaued at 12 nM. The granular cells remaining in suspension appeared more sensitive to higher concentrations of CTX than plasmatocytes since the degree of change in granular cell adhesion was more pronounced. Attachment levels of hemocytes from insects injected with CTB (0, 30, 60, 300 nM; Fig. 2.6B) showed similar patterns to the total number of adhering hemocytes and differential counts from insects injected with corresponding levels of CTX, albeit the changes in total hemocyte counts were not as pronounced implying hemocytes were less sensitive to CTB than CTX. CTA had no effect on the adhesive abilities of hemocytes from injected insects (Fig. 2.6C), which was anticipated because lacking CTB they would probably not enter the hemocytes.

Since the addition of hemocytes to glass was not standardized for the previous experiments, ratios of total circulating hemocytes from insects injected with CTX, CTB and CTA (see Fig. 2.5) to total attached hemocytes from the corresponding treatments and concentrations (Fig. 2.6A,B,C) were used to determine the adhesive properties of the hemocytes. Hemocytes from insects injected with 0, 12, and 60 nM CTX showed similar adhesion capabilities to glass (Fig. 2.6D), despite higher levels of circulating cells in insects injected with 12 and 60 nM CTX (see Fig. 2.5). The release of hemocyte stores during infection increases the number of hemocytes available in the hemolymph, these hemocytes participating in immune reactions (Gagen and Ratcliffe, 1976). Increased circulation of adhering hemocytes in CTX-injected larvae might reflect the infection response *in vivo*, implying hemocyte mobilization. Hemocytes from insects injected with 6 nM CTX showed a significantly lower level of adhesion ($p < 0.05$), concurrent with the elevated levels of circulating hemocytes seen in the treated insects (see Fig. 2.5), implying the increase in circulating hemocytes was the result of loss of adhesion to surrounding tissues. The adhesive properties of hemocytes from insects injected with CTB were the same between 0, 30 and 60 nM, after which a small decrease in adhesion was seen in hemocytes from 300 nM CTB-injected insects (Fig. 2.6D). The adhesive properties of hemocytes from insects injected with CTA were the same at all concentrations (Fig. 2.6D), and since CTA had no effect on the concentration of circulating hemocytes (see Fig. 2.5) this suggests its inability to breach the hemocyte surface.

Effect of cholera toxin on bacterial removal and nodule formation

Since CTX caused hemocytes to enter the circulation, similar to mobilization during bacterial infection, I examined CTX's effect on bacterial removal from the larval hemocoel. Insects concomitantly injected with *Ba. subtilis* and CTX more easily removed bacteria from circulation than insects injected with PBS only (Fig. 2.7). Insects injected with 6 nM CTX significantly removed (30%; 15.8-21.9; $p < 0.05$) more bacteria than the control, with 12 nM CTX significantly removing (15%; 6.6-9.0; $p < 0.05$) also more *Ba.*

subtilis than PBS only, but less than 6 nM CTX. Insects injected with 60 nM CTX removed the greatest number of bacteria (55%; 26.4-32.8; $p < 0.05$) compared to the PBS control (Fig. 2.7). The lowest concentration of injected CTB (30 nM) had no effect on bacterial removal, however, subsequent concentrations lowered circulating bacteria in a dose-dependent manner to levels significantly lower (64%; 35.8-39.3; $p < 0.05$) than the PBS control (Fig. 2.7). Increasing concentrations of CTA had no effect on bacterial removal (Fig. 2.7), implying again that individual CTA subunits cannot enter the hemocytes without CTB. CTB at high concentrations enhanced bacterial removal statistically similar to CTX ($p > 0.05$) indicating CTB may explain higher CTX results similar to results *in vitro*, further supporting an immunological role for CTB at higher concentrations.

The ability of CTX to mobilize and enhance the removal of bacteria may indicate its influence on nodule formation. The amount of nodules in the hemocoel of insects injected with CTX only was determined 24 h post-injection since nodules are more easily visible after having melanised by this time (Ratcliffe and Gagen, 1977). Nodule frequency significantly increased (41%; 22.1-27.6; $p < 0.05$) by 6 nM CTX, followed by a significant decrease (37%; 17.8-24.6; $p < 0.05$) by 12 nM and an increase by 60 nM to levels similar to 6 nM ($p > 0.05$; Fig. 2.8A). There was a significant negative correlation between the remaining circulating bacteria and the frequency of nodule-like structures from insects injected with CTX ($R = -0.95$; $p < 0.05$), suggesting CTX enhances bacterial removal by inducing nodulation. That hemocytes from larvae injected with 6 nM CTX had impaired adhering ability to glass, but induced nodule formation, reflects my *in vitro* results wherein 1.2 nM (corrected for tissue dilution) reduced hemocyte-glass adhesion but induced microaggregation. Higher nodule frequency at 12 and 60 nM CTX also reflects increased microaggreation observed *in vitro*. The nodule-like structures found in CTX-injected insects (Fig. 2.8B) bound to surrounding tissues and melanised similar to nodules in *Ba. subtilis*-injected insects (Fig. 2.8C), implying similar immune processes.

DISCUSSION

Although G proteins can stimulate cecropin B gene expression in the *Drosophila* mbn-2 hemocyte cell line (Samakovlis *et al.*, 1992), little is known about their involvement in hemocyte adhesive behaviours involving hemocyte-substratum (including mobilization which can involve hemocytes adhering to tissue surfaces) and hemocyte-hemocyte adhesion. These innate cellular immune responses are essential for the removal of microorganisms from the insect circulation. The salient hemocyte adhesion-based responses include phagocytosis and nodulation (Ratcliffe and Rowley, 1974; Ratcliffe and Gagen, 1977), of which the latter is influenced by microbe type and number (Gagen and Ratcliffe, 1976). Nodulation is a biphasic response composed of the hemocyte types the granular cells and plasmatocytes, the former being surveillance cells that react to foreign materials and release proteins that activate the plasmatocytes and mediate interactions between the immunocyte types (Gardiner and Strand, 1999; Strand and Clark, 1999; Nardi *et al.*, 2005). Plasmatocytes attracted to the granular cell-microbe coagulum, form layers that effectively quarantine most microorganisms (Ratcliffe and Gagen, 1977; Pech and Strand, 1996).

Hemocyte adhesion describes two main types of non-self responses: the initial hemocyte-substratum/microbe response and hemocyte-hemocyte interactions during multicellular immune reactions, such as nodulation. Distinguishing between the two types of interactions is crucial in understanding both the spatial and temporal organization of early and late cellular immune responses and their associated transduced signals. Hemocyte-substratum adhesion, including hemocyte-glass and hemocyte-microbe, and hemocyte-hemocyte interactions occur after non-self recognition. Recognition of microbes and their molecular antigens may represent interactions between plasma proteins, such as apolipophorin III, immulectin-2, hemolin, β1,3-glucan recognition protein and noduler, and foreign materials which then mediate hemocyte activities (Ma and Kanost, 2000; Yu and Kanost, 2002; Whitten *et al.*, 2004; Yu *et al.*, 2006; Gandhe *et al.*, 2007). Plasma-independent recognition receptors exist also on hemocytes triggering non-self activities (Dunphy and Nolan, 1980; Lackie, 1983; Strand and Lavine, 2001). After recognition, arthropodan granular cells discharge extracellular matrix (ECM)

proteins (Ball *et al.*, 1987; Johansson and Söderhall, 1988; Liang *et al.*, 1992; Scapigliati *et al.*, 1997; Holmblad and Söderhall, 1999), which faciliate hemocyte-microbe adhesion (Lamprou *et al.*, 2005) and hemocyte-hemocyte interactions (Nardi *et al.*, 2005). Additionally, granular cell ECM proteins elicit plasmatocyte activation (Strand and Clark, 1999; Nardi *et al.*, 2006). The eicosanoid prostaglandins (PG), derived from arachidonic acid by cyclooxygenase, are released from hemocytes due to non-self activation and enhance microaggregation (protonodules) possibly by elevating cAMP after binding to its G protein-coupled receptor (GPCR; Miller and Stanley, 2001; Stanley and Miller, 2008).

That CTX influences hemocyte activities is based on (1) the bimodal adhesion response of hemocytes to glass, (2) bimodal changes in total hemocyte counts *in vivo*, and (3) hemocyte-hemocyte contact with the formation of *in vitro* microaggregates but unlike the former two results, the aggregates did not adhere to slides at lower concentrations of CTX. The implication is that, depending on physiological concentrations of CTX, the bimodal effect on the hemocyte responses is influenced by $G\alpha_s$ or an unknown CTB-related signaling event, the former produced by altering levels of intracellular cAMP. Intracellular cAMP in insect fat body and endocrine glands is elevated by pharmacological levels of CTX (Meller *et al.*, 1990; Vroemen *et al.*, 1995; Arrese *et al.*, 1999), whereas we've shown that CTX can have physiological effects at much lower concentrations. cAMP-related hemocyte activities have been described in several insect species, in which adhesion to glass and bacteria by *G. mellonella* and *Malacosoma disstria* hemocytes is mediated by low cAMP while high concentrations impair adhesion (Marin *et al.*, 2005; Gulii *et al.*, 2009). The low adhesion reported in these studies may reflect hemocytes washed away as individual cells, but based on the present results, likely reflects hemocyte removal by way of rinsed off microaggregates. Low cAMP, as opposed to high cAMP, impairs also bacterial phagocytosis in hemocytes of *G. mellonella* (Brooks and Dunphy, 2005) and *Periplaneta americana* (Baines and Downer, 1992) and possibly nodulation in *Ma. sexta* (Stanley and Miller, 2006).

CTX in monkey Vero and BSC-1 epithelial cells and mouse embryonic fibroblastic cells crosses the plasma membrane after the AB_5 toxin binds to the GM1 ganglioside on the outer leaflet by means of the B-subunit (Bastiaens *et al.*, 1996; Chinnapen *et al.*, 2007). Within 5-10 min, CTX is internalized and fragments into the

toxic A-subunit where, by retro-translocation, it enters the cytosol from the endoplasmic reticulum (Bastiaens *et al*., 1996). CTB binding to *Ma. sexta* blood cells (Nardi *et al.*, 2005) implies, as does my work with CTX and CTB, that insect hemocytes may possess GM1-like gangliosides associated with lipid rafts. CTB activates the Th2 type of immune responses of both human dendritic cells and T cells (Holmgren *et al.*, 2005) and augments vaccine efficacy as an adjuvant when conjugated to antigens thus diminishing antigen levels required for T cell activation (George-Chandy *et al.*, 2001). Herein described for the first time, the physiological effects of high concentrations of CTB on insect hemocytes consist of increased hemocyte microaggregation *in vitro* and increased removal of *Ba. subtilis* from the hemolymph *in vivo*, while having no effect at low concentrations.

Control and CTX-induced *in vitro* microaggregates adhered to glass display rosetting plasmatocytes around their edges. Similar cellular composition occurs for stimulated lepidopteran larval hemocytes *in vivo* and *in vitro* (Ratcliffe and Gagen, 1977; Davis and Preston, 1985). Based on plasmatocyte directional movement indicated by the spread of the lobopodium (Davies and Preston, 1985), the plasmatocytes of *G. mellonella* were predominantly moving away from the *in vitro* microaggregates. The plasmatocytes in lepidopteran larvae of *Antheraea polyphemus*, *Samia cynthia*, and *Hyalophora cecropia* appear to immobilize the *in vitro* microaggregate (Walters, 1970). Plasmatocytes are incorporated also into microaggregates during early phase nodule formation (Ratcliffe and Gagen, 1977), and plasmatocytes from *Ephestia kuehniella, Pieris brassicae* and *Aglais urticae* exomigrate from *in vitro* microaggregates from areas of high hemocyte density to areas of low hemocyte density (Davies, 1983; Davies and Preston, 1985). The plasmatocyte migration function in the present study is unknown and to some extent unexpected because plasmatocytes usually wall off nodules (Ratliffe and Gagen, 1977). Hemocytes and epithelial cells of larval *Pseudaletia separata* produce an ENF family protein, the hemocyte chemotactic peptide, which induces both hemocyte migration to the chemokine source and hemocyte microaggregation (Nakatogawa *et al*., 2009). Other ENF family members, such as the plasmatocyte spreading peptide and paralytic peptide in *Ps. includens*, *Ma. sexta*, and *Pse. separata* are involved in

plasmatocyte spreading and aggregation (Strand and Clark, 1999; Wang *et al.*, 1999; Nakatogawa *et al*., 2009).

Both CTX and CTB triggered hemocyte-hemocyte adhesion *in vitro* and CTX, and presumably CTB, induced adhesion *in vivo* in the form of nodules at high treatment levels. This was anticipated because CTX agglutinates liposomes and sheep erythrocytes by binding to GM1 gangliosides (Richards *et al*., 1979) due to CTB cross-linking the ganglioside on lipid rafts between adjacent Teff cells (Wang *et al.*, 2009). Although Nardi *et al*. (2005) established CTB binding to live granular cells of *Ma. sexta*, agglutination was not detected, which may reflect the toxin concentration being too low to elicit cell-cell adhesion.

Insect blood cells exist in three compartments: floating in the hemolymph, attached to various organs and tissues, and beneath the epidermis (Wigglesworth, 1956; Mangalika *et al*., 2010; Kim and Kim, 2009; Markus *et al*., 2009) where they immediately mobilize in response to trauma and infection resulting in an increase in hemolymph blood cells coinciding with the initiation of nodulation (Gagen and Ratcliffe, 1976; Ratcliffe and Gagen, 1977). Our results show that CTX causes mobilization of hemocytes as indicated by similar proportions of adhesive hemocytes from CTX- and PBS-injected larvae, despite larger increases in circulating hemocytes in the former group. In *Sp. exigua*, the hemocyte type most predominantly mobilized 4 h after *Es. coli* injection is the granular cell (Kim and Kim, 2009) and may reflect a similar event after CTX-injection in *G. mellonella* initiating the microaggregation event. This further supports the granular cells' primary role as immune surveyor cells (Gardiner and Strand, 1999; Strand and Clark, 1999; Nardi *et al.*, 2005).

The RGDS tetrapeptide inhibited *in vitro* CTX-induced microaggregation of *G. mellonella* hemocytes in a concentration-dependent manner reducing hemocyte content per microaggregate, whereas RGES did not. RGDS did not dissociate or lyse hemocytes previously attached to slides. Thus, the effect on *in vitro* microaggregates was not likely due to the dissociation of hemocyte-hemocyte interactions or lysis. RGD receptors on hemocytes are involved with adhesion to extracellular matrix proteins (ECM) influencing hemocyte spreading in molluscs (Plows *et al.*, 2006), crayfish hemocyte degranulation (Johansson and Söderhall, 1989), and microparticulate phagocytosis by lepidopteran

(Wittwer and Wiesner, 1996), dipteran (Foukas *et al.*, 1998), and bivalve (Plows *et al.*, 2006) hemocytes. However, the RGD effects may vary with the insect species/assay method. RGD has no effect on *Sp. littoralis* hemocyte dissociation from coverslips or *Es. coli* adhesion to hemocyte monolayers (Costa *et al.*, 2005). These results may reflect maximum three dimensional ECM discharge around the hemocytes precluding RGD interference with integrins; in which case bacteria adhered to the ECM by an RGD-independent mechanism. That is not to say that *Es. coli* does not react with integrins, its outermembrane protein, intimin, reacts with β_1 epithelial integrins (Frankel *et al.*, 1996). Adhesion of freely floating *Ps. includens* hemocytes to polystyrene tissue culture plates exhibits RGDS dose-dependent inhibition (Pech and Strand, 1995). Medfly, *Ceratitis capitata* hemocytes phagocytose both Gram-positive and -negative bacteria by an RGD-dependent mechanism but phagocytosis of latex beads is RGD-independent (Lamprou *et al.*, 2007).

That the CTX-induced *in vitro* microaggregate reaction in *G. mellonella* was RGDS-related implies integrins are involved. The influence of G proteins on integrin-related processes is not unexpected since $G\alpha_{13}$ directly associates with the cytoplasmic tail of the β_3 integrin in mouse platelets to mediate cell spreading (Gong *et al.*, 2010). Integrins on invertebrate hemocytes have been reported for the two major phyla, P. Mollusca [e.g. the Pacific oyster, *Crassostrea gigas* (Terahara *et al.*, 2006)] and P. Arthropoda, especially insects (Lavine and Strand, 2003; Levin *et al.*, 2005; Zhuang *et al.*, 2007b; Zhuang *et al.*, 2008). The types of hemocyte integrins, whose nomenclature is unrelated to mammalian integrins, vary in distribution with the hemocyte type and insect species (Lavine and Strand, 2003; Zhuang *et al.*, 2008). PGE_2, which stimulates *G. mellonella* and *Mal. disstria* hemocyte adhesion (Marin *et al.*, 2005; Gulli *et al.*, 2009), may influence integrin-generated activity, as it activates integrin-dependent adhesion and spreading of human endothelial cells (Dormond *et al.*, 2002). Both α and β integrin subunits participate in encapsulation in lepidopteran larvae (Wiegand *et al.*, 2000; Lavine and Strand, 2003; Zhuang *et al.*, 2008). That nodulation is similar to encapsulation (Ratcliffe and Gagen, 1977), it is likely that nodulation is integrin-mediated.

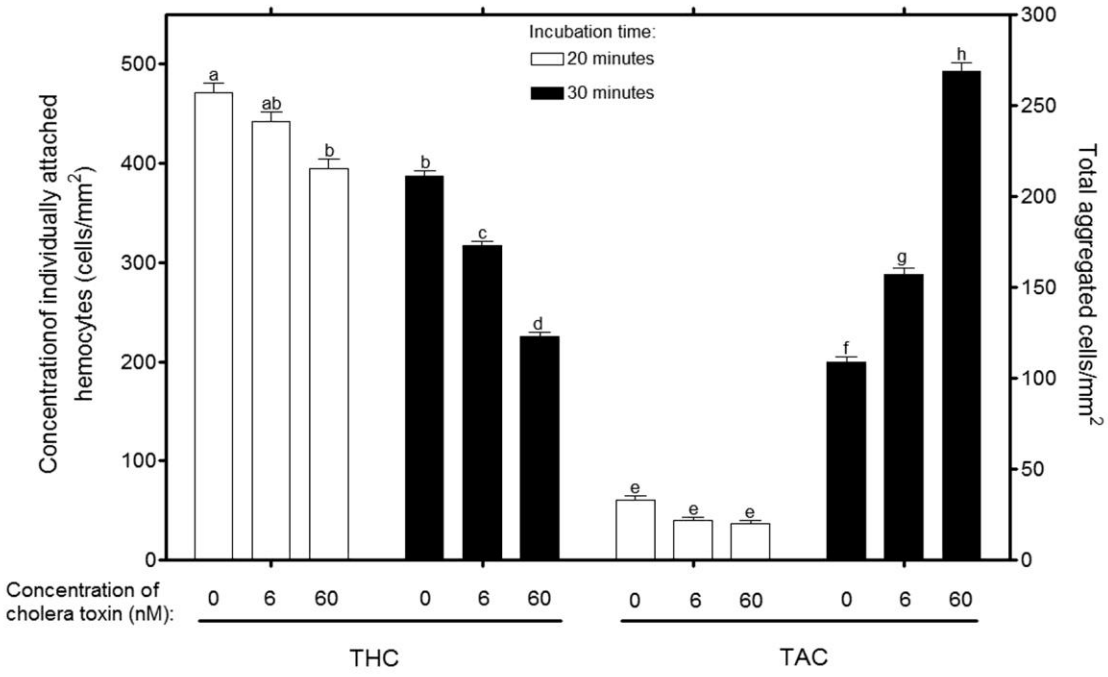

Figure 2.1. Thirty minutes is the most effective incubation time to treat hemocytes with whole cholera toxin *in vitro*.
Hemolymph suspensions were added to slides containing increasing concentrations of soluble CTX and the total individual and aggregated hemocytes determined after 20 and 30 minutes incubation. THC = total individual hemocyte count; TAC = total aggregated cells. Bar values with the same letter on the top are not significantly different ($P > 0.05$)

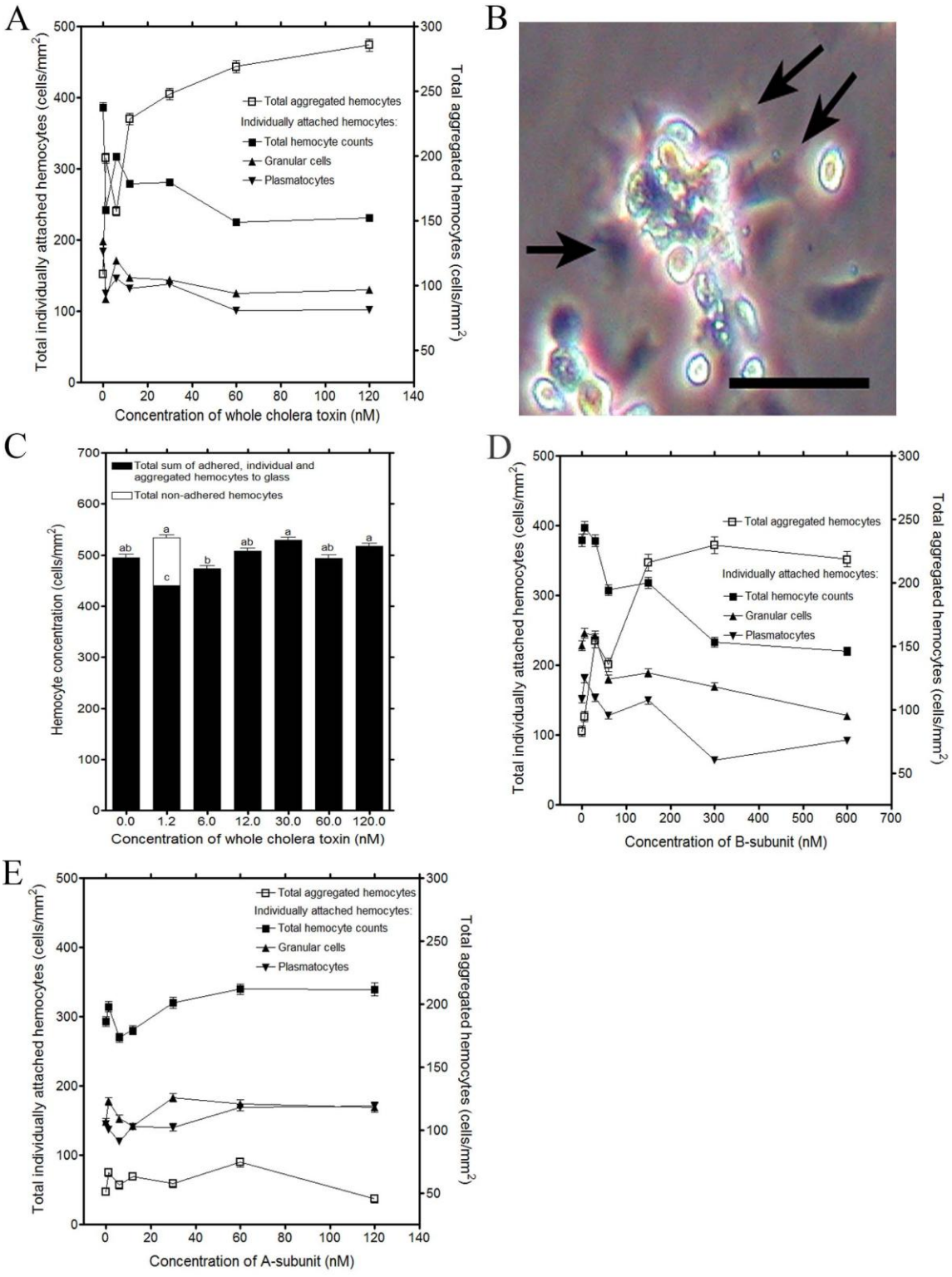

Figure 2.2. Whole cholera toxin and its B-subunit modulates hemocyte adhesion to glass slides and induces *in vitro* microaggregation.

Hemolymph suspensions were added to slides containing increasing concentrations of soluble CTX (A), producing microaggregates attached to a monolayer of plasmatocytes (arrows) arranged in a rosetting pattern around the coacervate (B; Scale bar represents

50 μm). Total adhered, individual and aggregated hemocytes (black bar; C) are equal throughout the concentrations of CTX except at 1.2 nM. The number of non-adhered hemocytes (white bar) from monolayers treated with 1.2 nM CTX was determined and added to total adhered hemocytes. Hemocytes were treated also with B-subunit (D) or A-subunit (E) (both at levels corresponding to CTX) and the total individual and aggregated hemocytes determined. Bar values with the same letter on the top are not significantly different ($P > 0.05$).

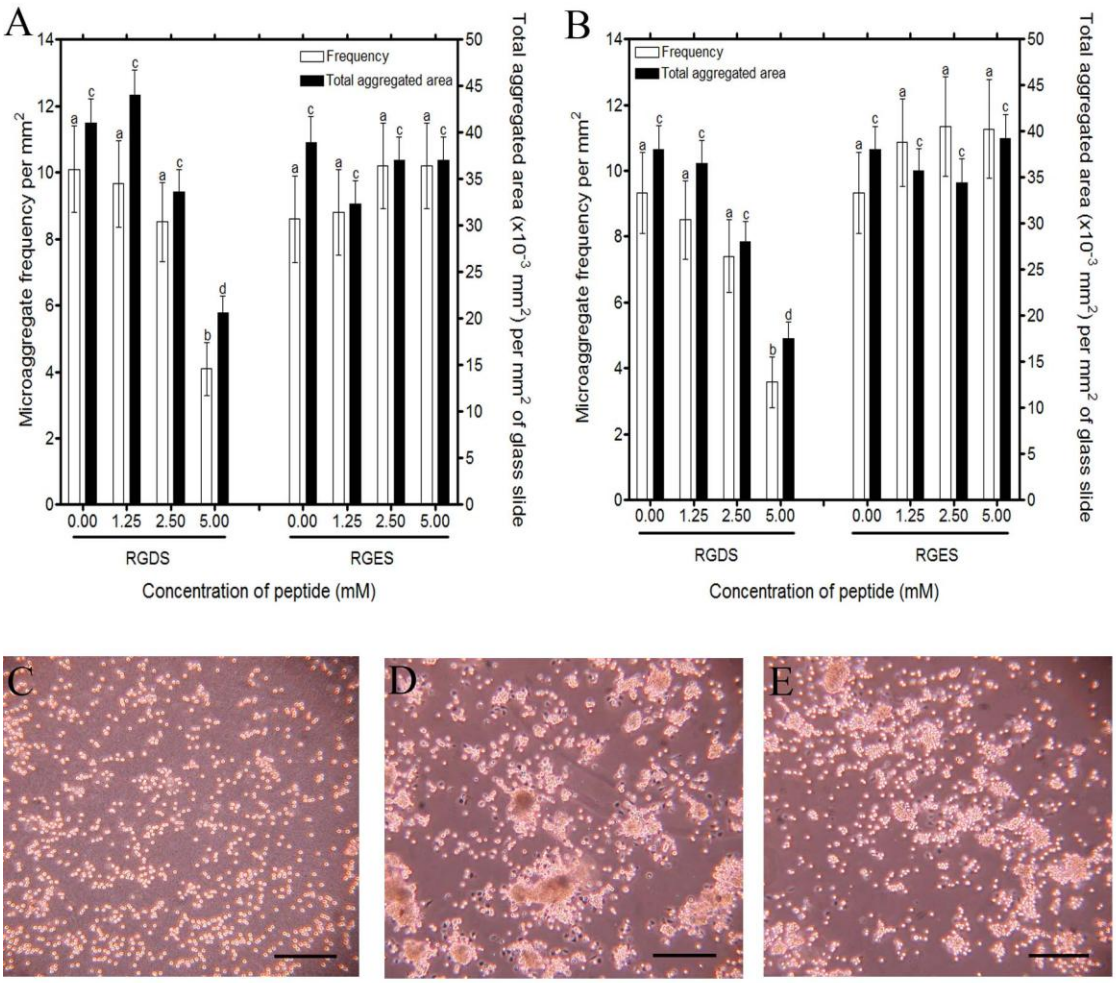

Figure 2.3. RGD peptides inhibit cholera toxin-induced microaggregate formation *in vitro*.

Hemolymph suspensions were added to slides containing 1.2 nM (A) or 120 nM (B) soluble CTX and increasing concentrations of soluble RGDS or RGES peptide. RGDS, but not RGES, inhibits aggregate frequency and total aggregate area. Images of hemocytes treated with CTX (1.2 nM) with 5 mM RGDS (C), without RGDS (D) and with 5 mM RGES (E) were taken with phase contrast microscopy. Scale bars represent 200 μm. Bar values with the same letter on the top are not significantly different ($P > 0.05$).

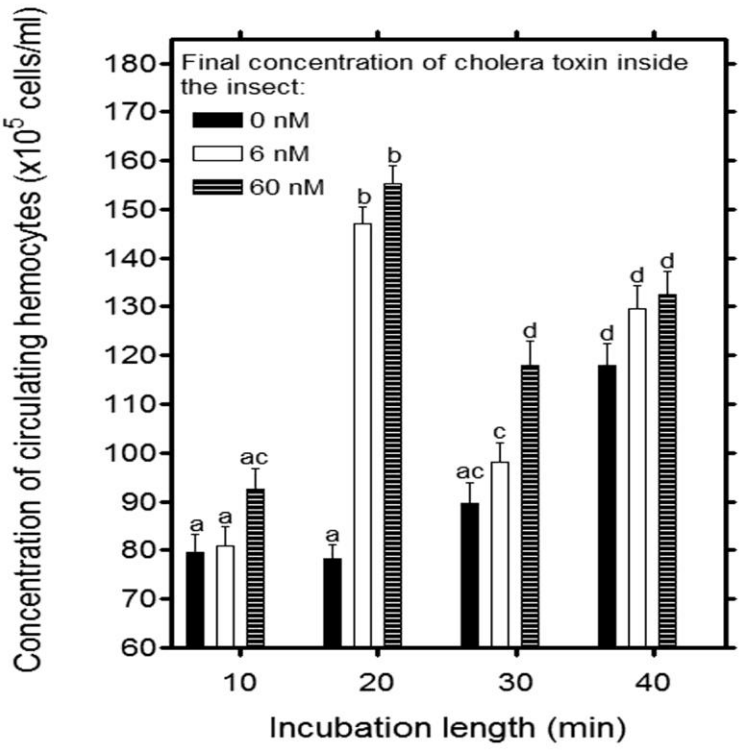

Figure 2.4. Twenty minutes is the most effective incubation time for injecting insects with whole cholera toxin.

Insects were injected with final concentrations of 0, 6, and 60 nM CTX and incubated for 10, 20, 30, and 40 min. The concentration of circulating hemocytes was then determined. Bar values with the same letter on the top are not significantly different ($P > 0.05$).

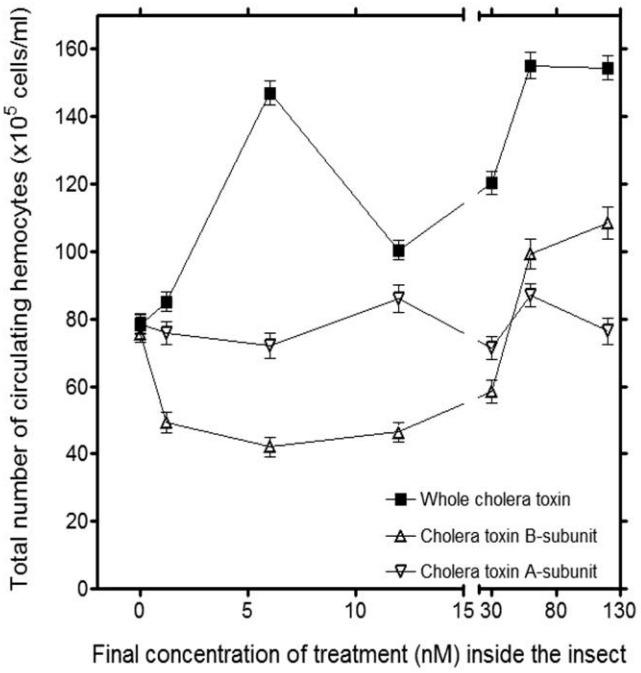

Figure 2.5. Effect of whole cholera toxin, and its A- and B-subunit components on the concentration of circulating hemocytes *in vivo*.
Insects were injected with CTX, CTA or CTB (molar equivalents corresponding to CTX: 0, 6, 30, 60, 150, 300, 600 nM) and the concentration of circulating hemocytes determined. CTB contributes to the physiological bimodal effect of CTX.

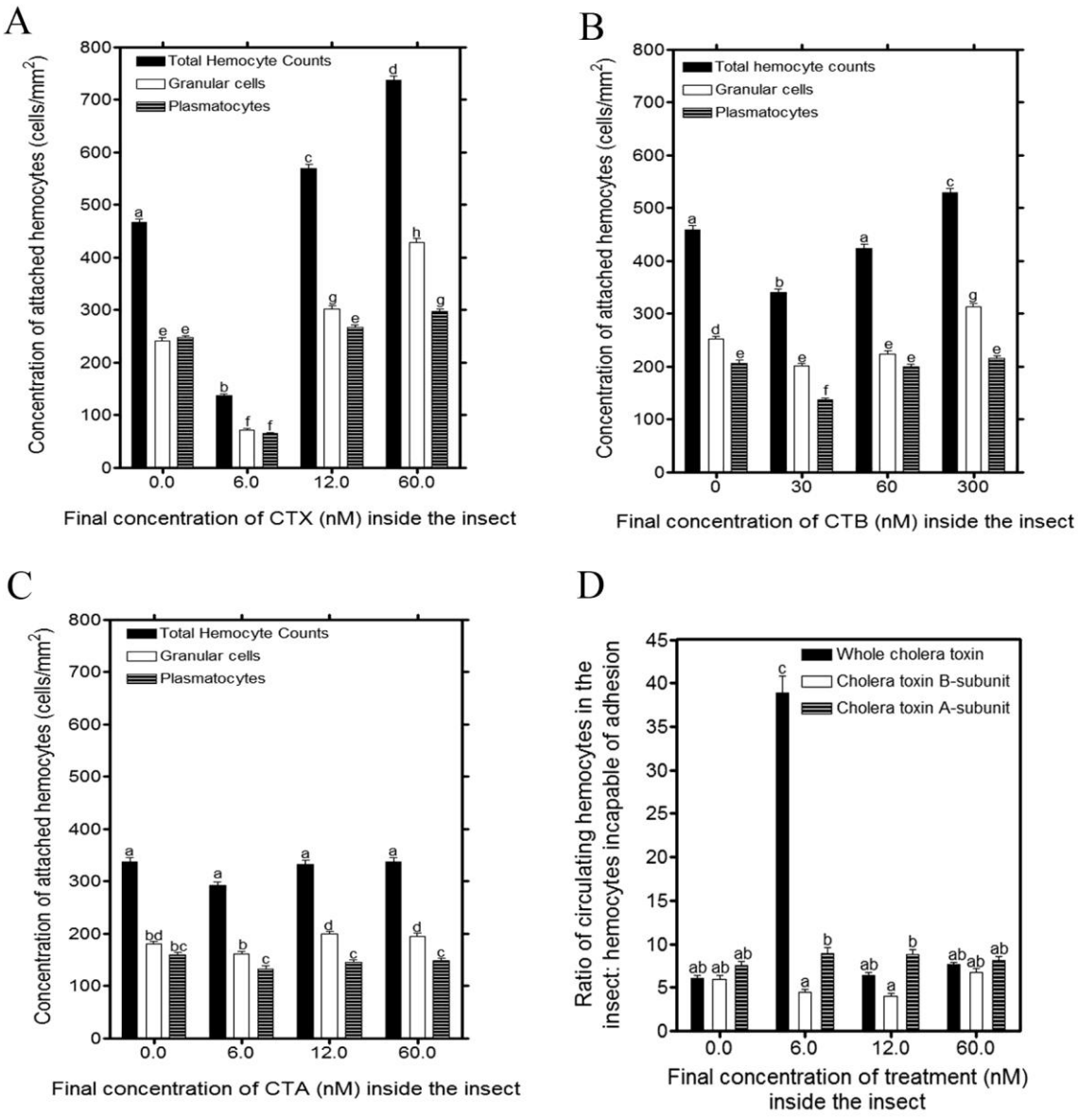

Figure 2.6. The adhesive properties of hemocytes from insects injected with whole cholera toxin or its individual components, A- and B-subunits.
Hemolymph (10 µl) was removed from insects injected with CTX (A) or molar equivalents (corresponding to CTX) of CTB (B) or CTA (C) and was added to glass slides. The number of attached hemocytes was then recorded. Ratios (D) of total circulating hemocytes from insects injected with CTX, CTB and CTA (see Fig. 2.5) to total attached hemocytes from the corresponding treatments and concentrations (Fig. 2.6A,B,C) were used to determine the adhesive properties of the hemocytes. Bar values with the same letter on top are not significantly different ($P > 0.05$).

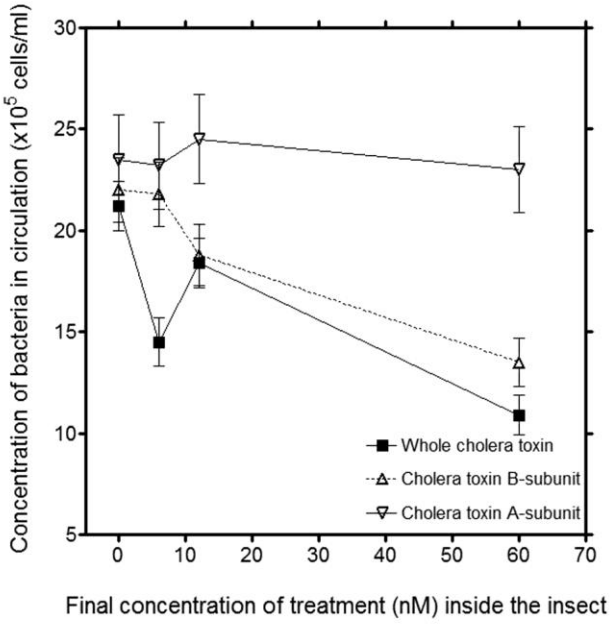

Fig. 2.7. Effect of whole cholera toxin and its A- and B-subunit components on the removal of bacteria from the hemocoel.
CTX and CTA (0, 6, 12, 60 nM) and corresponding levels of CTB (0, 30, 60, 300 nM) were concomitantly injected with *Ba. subtilis* into the insect hemocoel and incubated for 20 min. The number of bacteria left in circulation was then recorded.

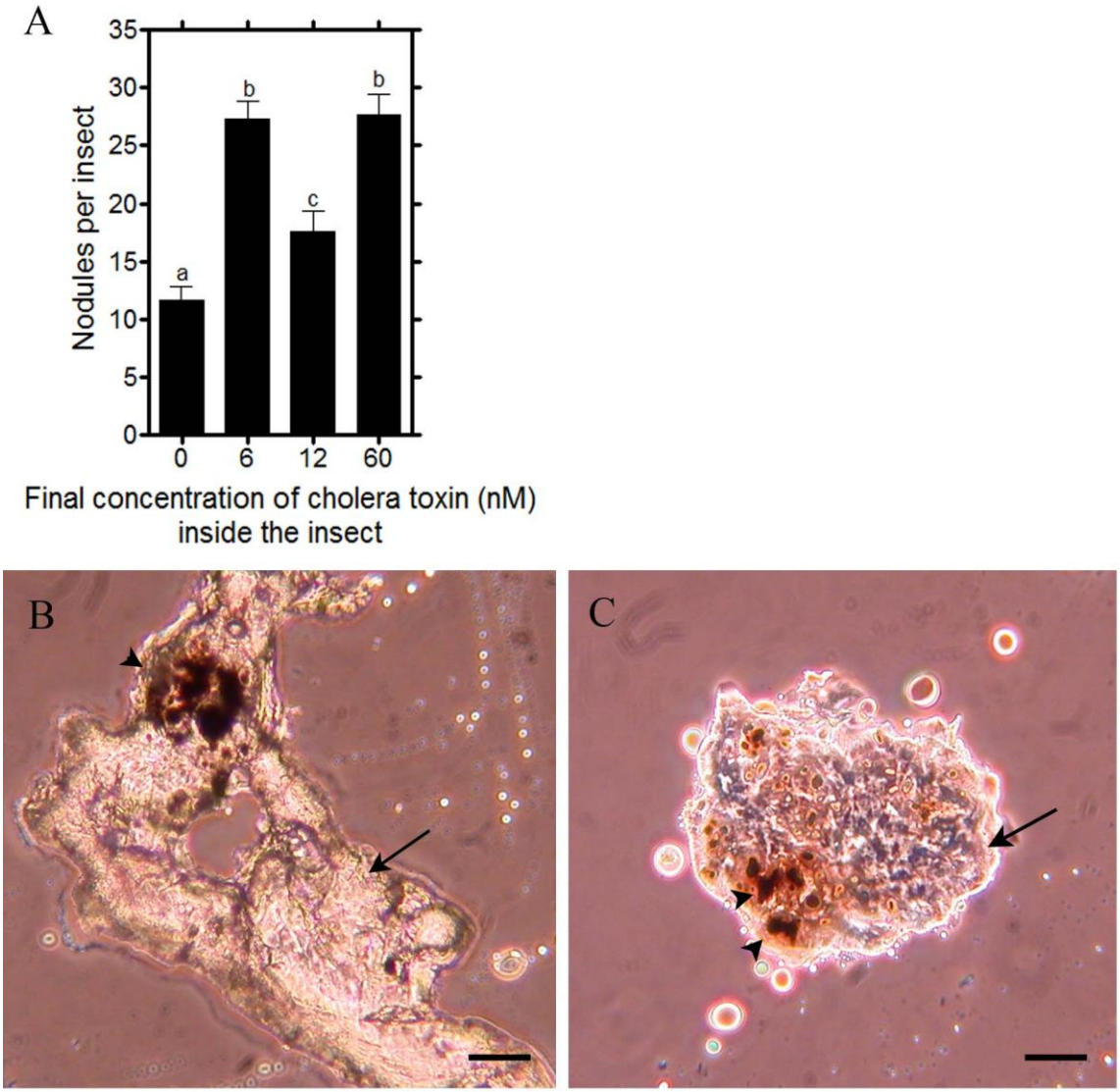

Figure 2.8. Effect of whole cholera toxin on nodule formation *in vivo* 24 h post-injection.

(A) Insects were injected with 0, 6, 12, and 60 nM CTX and the number of melanised nodule-like structures in the hemocoel was recorded. Bar values with the same letter on top are not significantly different ($P > 0.05$).

(B) Image of a CTX-induced nodule-like structure (arrowhead) attached to the fat body (arrow).

(C) Image of a *Ba. subtilis*-induced nodule (arrowhead) attached to fat body tissue (arrow). Scale bars of (B) and (C) represent 50 μm.

CHAPTER 3: The involvement of integrin-like molecules in the cellular immune responses of *G. mellonella* hemocytes

PREFACE

The insect immune system is simpler than its mammalian counterpart in that it has an innate immune response only (Hoffman, 2003). Insect and mammalian innate immunity shares similar overall responses and signal transduction events (Kemp and Massey, 2007). Previously, in chapter 2, I established the relationship between whole cholera toxin and *in vitro* hemocyte microaggregation in *Galleria mellonella*, the latter being inhibited by an RGDS peptide suggesting the involvement of integrins. Integrins mediate cell-cell adhesion interactions throughout the animal kingdom (Hynes, 2002). Their involvement in the multi-cellular immune response of nodulation in lepidopteran insects has been alluded to in past research (Nardi *et al.*, 2005; Zhuang *et al.*, 2007b). Herein (Chapter 3), I characterized the surfaces of select immune reacting hemocyte types with integrin antibodies and monitored the immuno-inhibitory effects of these antibodies on the hemocyte immune response(s) to bacteria. Furthermore, I observed an increase in integrin labeling between interacting hemocytes, granular cells and plasmatocytes, with increasing cholera toxin.

ABSTRACT

Insect blood cell-blood cell (i.e. hemocyte-hemocyte) interactions are crucial in containing large numbers of unicellular microbes and larger multicellular microorganisms during nodulation and encapsulation, respectively. Hemocyte-substratum and hemocyte-hemocyte interactions form the basis of the cellular immune responses of phagocytosis, nodulation, and encapsulation. Integrins play a crucial role in cellular adhesion across the metazoan kingdom, and the extracellular matrix proteins discharged from the insect hemocyte types, the granular cells, are known to participate in integrin-mediated hemocyte-microbe and hemocyte-hemocyte adhesion. Herein, I used four human integrin antibodies capable of labeling *Galleria mellonella* hemocyte surfaces and recognizing potentially integrin-like molecules on hemocytes. The β_1 and α_5 integrin subunit antibodies not only labeled plasmatocytes and granular cells, but were also capable of preventing bacterial removal from the hemolymph when injected with bacteria into the insect hemocoel. The α_5 integrin subunit antibody bound a molecule of similar molecular weight (145 kDa) as its human counterpart and also intensely stained the junction between interacting granular cells and plasmatocytes; the frequency of this fluorescent band increased with increasing cholera toxin treatment. Granular cells and plasmatocytes in *in vitro* microaggregates formed with and without cholera toxin displayed a similar hemocyte arrangement (a cluster of granular cells atop a monolayer of plasmatocytes) despite differences in size. The structure of the *in vitro* microaggregate may be analogous to early nodule formation *in vivo*.

INTRODUCTION

Insect hemocytes possess several immune mechanisms similar to vertebrates, such as the respiratory burst pathway (Bergin *et al.*, 2005; Renwick *et al.*, 2007), and adhesion- (Bryant and Sutcliffe, 1974; Marin *et al.*, 2005) and phagocytosis-related signaling components (Mócsai *et al.*, 2000; Rubel *et al.*, 2002; Lamprou *et al.*, 2007), and have garnered much interest in their use as immune models (Hoffmann, 2003). Devoid of the acquired immune responses typically found in vertebrates, all insect orders examined to date contain representatives possessing only innate immunity comprised of interactive cellular and humoral components (Gillespie *et al.*, 1997; Hoffmann, 2003). After immune recognition, humoral responses include the early activation of the phenoloxidase cascade (Söderhall and Cerenius, 1998), and the later synthesis of antimicrobial peptides from the epithelium, fat body and hemocytes (Ferrandon *et al.*, 1998). The immediate cellular response involves engagement of the hemocytes in particulate microbial removal by way of hemocyte-antigen adhesion followed by phagocytosis and/or nodulation (Gillespie *et al.*, 1997). Both types of cellular immune responses can be mediated by constitutive humoral factors acting as pattern recognition proteins and/or opsonins (Dunphy and Halwani, 1997; Ma and Kanost, 2000; Yu and Kanost, 2002; Dziarski and Gupta, 2006; Gandhe *et al.*, 2007; Lee, 2007), but cellular responses are known to occur independent of humoral influence in both insects and mammals (Dunphy and Nolan, 1980; Lackie, 1983; Ofek *et al.*, 1995).

Nodulation is a biphasic cellular immune response towards large numbers of small microorganisms, such as bacteria, yeast and certain stages of fungal development (conidia and spores), implemented by the phase bright, spherical granular cells and phase dark, amoeboid plasmatocytes (Ratcliffe and Gagen, 1976; Ratcliffe and Gagen, 1977; Lord *et al.*, 2002). During the first phase of nodulation granular cells contact the microorganism releasing adhesion molecules creating a mass of sticky coagulum, entrapping the microbes, and other granular cells and some plasmatocytes; these microaggregations subsequently adhering to surrounding tissues (Ratcliffe and Gagen, 1977). The second phase begins as chemoattractants discharged by granular cells recruit plasmatocytes which adhere to and spread on the microaggregates forming a multilayered

nodule that is eventually removed from the insect's hemocoel (Ratcliffe and Gagen, 1977). The function, formation and structure of insect nodules resembles that of granulomas and capsules in molluscs (Sminia *et al*., 1974; Harris and Cheng, 1975; Sangster and Smolowitz, 2003), annelids (Cooper, 1968; Porchet-Henneré *et al.*, 1987), and mammals (Majno and Joris, 2004).

Little is known about the molecular mechanisms of lepidopteran nodulation, however, studies *in vitro* can compartmentalize both phases of nodulation, while research on a related immune response, encapsulation, can also continue to provide insight (Schmit and Ratcliffe, 1977). Noduler, a protein capable of binding hemocytes and the associated molecular antigens of microorganisms likely mediates microaggregation during the initial stage of nodulation (Gandhe *et al.*, 2007). C-type lectins isolated from the larval hemolymph of the lepidopteran silkworm, *Bombyx mori* mediate nodulation by binding Gram-negative and Gram-positive bacteria and fungi (Koizumi *et al.*, 1999; Watanabe *et al.*, 2006). Subsequent stages of nodulation involve hemocyte-hemocyte interactions. The extracellular matrix protein lacunin and a peanut agglutinin lectin-reactive ligand in granular cells are released upon non-self recognition, the proteins coating adjacent hemocytes enabling hemocyte-hemocyte interactions by means of integrin receptor clustering (Nardi *et al.*, 2005). Transmembrane proteins, such as tetraspanin, mediate also hemocyte-hemocyte interactions via integrins (Zhuang *et al*., 2007b). Other hemocyte surface proteins, such as neuroglian, will bind integrins on adjacent hemocytes to mediate interactions between granular cells and the plasmatocytes forming focal points for hemocyte aggregation (Zhuang *et al.*, 2007a), suggesting neuroglian may be responsible for adhering the aggregating granular cells to plasmatocytes. The latter hemocyte type is known to be the first hemocyte type to settle onto lepidopteran (specifically *Galleria mellonella*) and phasmid insect tissues during immune stimulation prior to microaggregates settling onto tissues *in vivo* (Chain and Anderson, 1982; Scapigliati and Mazzini, 1994). Plasmatocyte spreading factor, isolated from *Pseudoplusia includens*, can stimulate spreading *in vitro* and may be involved in plasmatocyte adhesion to and spreading on the microaggregate during nodulation (Clark *et al.*, 1997). Prostaglandins, cyclooxygenase-derived fatty acids, are known mediators of nodulation in response to bacterial and microsphere inoculation in *Manduca sexta* and *G*.

mellonella larvae, respectively (Miller *et al.*, 1994; Mandato *et al.*, 1997), however, a link between these eicosanoids and integrins has not been presented. The presence of precipitated hemolymph on the nodule may deter more plasmatocytes from attaching, terminating the nodulation event (Ratcliffe and Gagen, 1977). Unlike encapsulation (Pech and Strand, 1996), whether granular cells are involved in the terminal step of nodulation is unclear.

Integrins are an integral part of the hemocyte-hemocyte interactions of nodulation, and their importance in immunological processes across the animal kingdom is well known. Integrins are surface receptors composed of the non-covalently bound heterodimers α and β subunits, which provide the adhering link between the extracellular matrix and a cell's actin cytoskeleton, and are involved in outward-in and inside-out signal transduction (Hynes, 2002). Spreading of NIH-3T3 fibroblast cells is preceded by fibronectin-adhesion integrin-dependent translocation of small GTPases, such as Rac and Cdc42, to the plasma membrane (Del Pozo *et al.*, 2002). Furthermore, integrins interact with the focal adhesion proteins, vinculin and talin, linking them to the actin cytoskeleton in chicken fibroblasts (Horwitz *et al.*, 1986) culminating in focal adhesion maturation (Patridge and Marcantonio, 2006). The actin-based filopodial structures on *Bom. mori* granular cells are necessary to phagocytose foreign material (Wago, 1983b, 1984), however, the filopodia of hemipteran plasmatocytes are not always involved in phagocytosis (Borges *et al.*, 2008). Actin in insect hemocytes influences adhesion-based immune responses as exemplified by the fact that many successful insect parasites disrupt the actin cytoskeleton of hemocytes preventing encapsulation (Davies and Preston, 1987; Webb and Luckhart, 1994; Webb and Luckhart, 1996; Li, *et al.*, 2009).

cAMP and its downstream effectors have a bimodal effect on insect hemocytes, as in mammalian leukocyte immune activities, in which immediate/early adhesion reactions are stimulated by low levels of intracellular cAMP (Bryant and Sutcliffe, 1974; Marin *et al.*, 2005; Brooks and Dunphy, 2005; Gulii *et al.*, 2009). The late cellular immune responses, such as phagocytosis and multicellular reactions, are mediated by greater levels of intracellular cAMP in both insects and mammals (Wahl *et al.*, 1979; Baines and Downer, 1992; Stanley and Miller, 2006). Furthermore, early hemocyte-substratum adhesion events, in bivalves and lepidopterans (Chen and Bayne, 1995a; Diehl-Jones *et*

al., 1996), and late immune responses, such as dipteran phagocytosis and lepidopteran encapsulation (Davies and Preston, 1987; Foukas *et al.*, 1998; Pearson *et al.*, 2003), depend on actin remodeling. Preliminary research on the involvement of cAMP in actin polymerization (Gupta and Campenot, 1996) and its upstream effectors, such as small GTPases, in invertebrates remains inconclusive. Phosphorylation of p92, leading to human neutrophil adhesion on fibrinogen-coated surfaces, is determined by integrin-dependent actin polymerization (Takami *et al.*, 2001), whereas activation of the cAMP/PKA pathway works to inhibit Rho, a regulator of actin remodeling, to prevent this adhesion (Laudanna *et al.*, 1997). Whether similar signaling mechanisms exist in insect hemocytes is not clear.

To assess the role of integrins in *G. mellonella* hemocyte adhesion, I characterized the potential function and hemocytic distribution of several integrin-like molecules. I also provide a possible functional link between *in vitro* microaggregation induced by whole cholera toxin (a G protein modulator activating adenylate cyclase) and the integrin-like molecules. Furthermore, I have determined the morphological structure of the *in vitro* microaggregates via confocal microscopy and discuss its similarities to nodules.

MATERIALS AND METHODS

Insect culture

Galleria mellonella larvae were raised in 4.5 L jars at an ambient temperature of 28 °C under constant light conditions on a multigrain diet supplemented with glycerol and vitamins (Dutky *et al.*, 1962). Brood comb was generously supplied by Claude Vinet (Miel Claude Vinet, Vaudreuil-Dorion, Quebec). Fifth instar larvae weighing 250 ± 10 mg were used. Both *in vitro* and *in vivo* experiments were conducted at 37 ºC to mimic temperature conditions in honey bee colonies (Southwick and Heldmaier, 1987) and in artificially reared *G. mellonella* colonies (Buchmann and Spangler, 1991). *G. mellonella* stocks were genetically enhanced by breeding in disease-free feral insects collected from apiaries.

Bacterial culture

Stock cultures of the non-pathogenic, Gram-positive bacteria *Bacillus subtilis* (Boreal Biological, Mississauga, ON, Canada) were grown on Luria agar (1 g NaCl, 1 g yeast extract, 2 g bactotryptone, 3 g agar; 200 ml water) at 25 ºC and subcultured every fortnight.

For experimental purposes, *Ba. subtilis* was added to 5 ml Luria broth in 20 ml scintillation vials and incubated overnight (25 °C, 200 rpm on a horizontal gyratory shaker), as in the previous Materials and Methods in Chapter 2. One hundred µl of the bacterial suspension was added to 5 ml Luria broth in a new 20 ml scintillation vial and incubated for 2 h as previously described. Five ml of culture was added to flasks (500 ml) containing 200 ml Luria broth and bacteria were grown to midlog phase (OD_{660nm} = 0.75 A). Bacteria were pelleted (12,000xg, 15 min, 25ºC) and resuspended in 10 ml of phosphate-buffered saline (138 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2.8 mM KH_2PO_4, adjusted to pH 6.5 with 6N HCl; PBS) before being killed by ultraviolet light exposure (3 h). To ensure complete bacterial death, bacterial suspensions (30 µl) were plated onto Luria agar and incubated at 25ºC and 30°C. Bacteria were then centrifuge-

washed (12,000xg, 15 min, 25 ℃) three times in PBS (10 ml) and stored at -20 ℃. Dead bacteria provided antigen-stimulation devoid of metabolic activity (Alavo and Dunphy, 2004).

Reagents and Antibodies

Whole, inactivated cholera toxin (CTX; BioMol International) and anti-dog IgG (Sigma) were used. Phalloidin conjugated to Alexa Fluor® 568 and donkey anti-goat IgG and goat anti-rabbit IgG both conjugated to Alexa Fluor® 488 were obtained from Molecular Probes. Donkey anti-goat IgG and donkey anti-rabbit IgG both conjugated to alkaline phosphatase, and polyclonal antibodies to anti-human β_1 integrin subunit (raised against N-terminus), anti-human β_3 integrin subunit (raised against N-terminus), anti-human α_v integrin subunit (raised against N-terminus), and anti-human α_5 integrin subunit (raised against amino acids 840-943) were obtained from Santa Cruz Biotechnologies.

Hemocyte lysate

For immunoblotting with integrin antibodies, plasma-free, whole cell lysates, prepared from unseparated hemocytes, were used. Fifteen µl of hemolymph was pooled from each of 40-45 insects and added to 1 ml chilled anticoagulant (98 mM NaOH, 186 mM NaCl, 17 mM Na_4EDTA, 41 mM citric acid, pH 4.5; Mead *et al.*, 1986). Suspended hemocytes were washed free of plasma by centrifugation (325 g, 4 min) three times, the cells being resuspended in 1 ml chilled PBS after each wash. After washing, the cells were re-suspended in SDS [1% w/v PBS (200 µl)] containing 1 µl protease inhibitor cocktail [4-(2-aminoethyl) benzenesulphonyl fluoride, 104 mM; aprotinin, 0.08 mM; leupeptin, 2 mM; bestatin, 4 mM; pepstatin A, 1.5 mM and E.64; 1.4 mM (Sigma)]. Hemocytes were lysed by vigorous pipetting and vortexing (100% lysis), the resulting lysate was analysed for total protein (Bradford, 1976) and stored at -80 °C.

Dot and Western Blot

Dot blots were performed by adding protein (20 µg) from centrifuged hemocyte lysate (14,000g 2 min, RT) to unwetted 1 cm^2 nitrocellulose membranes (Millipore). Hemocytes lysates were centrifuged, prior to its addition to the membrane, in order to avoid the formation of an unidentified yellow precipate on the membrane which prevented antibody labeling. The membrane was then wetted with Towbin buffer [25 mM Tris-HCl, 192 mM glycine, 20% methanol (v/v), pH 8.2] and let dry on filter paper (BioRad). Five ml volumes of blocking, labeling, and washing solutions (described below) were used on one 1 cm^2 membrane. Subsequent steps were identical to Western blotting (described below). Dot blots were used to optimize conditions for the Western blot.

For Western blots, centrifuged hemocyte lysate protein (20 µg) dissvolved in Laemmli sample buffer (95 °C, 5 min) was loaded onto a 7.5% (0.75 mm) separating polyacrylamide SDS-gel and resolved at constant voltage (200 V). The 7.5% gel was chosen for its molecular weight resolution (40kDa – 300kDa) since the size of the four integrin subunits found in invertebrates range from 90kDa to 200kDa (Foukas *et al.*, 1998; Levin *et al.*, 2005; Terahara *et al.*, 2006). Separated proteins were transferred to nitrocellulose membrane (0.22 µm) in Towbin buffer (35 V, RT). Individual lanes were cut from the membranes and probed with a given antibody. Five ml volumes of blocking, labeling, and washing solutions (described below) were used on one lane.

For both dot and Western blotting, the membranes were blocked by incubating with 0.2% Tween-20 (v/v) with 3% BSA (w/v) in PBS for 1 h. Membranes were washed with three changes (5 min, 50 rpm on a horizontal gyratory shaker) of 0.2% Tween-20 (w/v) in PBS, and, after secondary antibody labeling, 0.2% Tween-20 (w/v) in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl; adjusted to pH 7.8 with 6 N HCl; TBS). The primary antibody [final concentrations of 0.2 µg/ml in 3% BSA (w/v PBS) for all integrin antibodies, except β_3 (0.4 µg/ml)] was incubated (50 rpm) with the membrane for 2 h (RT) or overnight (4 °C). Probed membranes were washed as previously described. Both secondary antibodies, anti-goat and anti-rabbit IgG conjugated to alkaline phosphatase (0.08 µg/ml), in 3% BSA (w/v in TBS) were incubated with the membrane under shaking conditions (1 h, 50 rpm, RT). Lumi-Phos WB Chemiluminescent Substrate (500 µl; Thermo Scientific) was added to the membrane and incubated for 35 min in darkness. Substrate was drained and the membrane analyzed with ChemiGenius

Chemiluminescence Image Analyser (Geneflow). Non-specific controls included incubating the membrane with the secondary antibody without primary antibody labeling. A minimum of three replicates was used.

Immunofluorescent labelling

Immunofluorescent staining was used to localize the integrin-like molecules on granular cells and plasmatocytes adhered to coverslips and also the distribution of the fluorescent pattern after CTX treatment. Hemocyte suspensions were made by collecting hemolymph (15 µl) from the third prothoracic proleg from each of six ice-chilled (10 min) larvae into chilled (4 °C) PBS (1.33 ml). Microcentrifuge tubes were gently inverted twice to prevent hemocyte activation and agglutination and adhesion to the tube surface. Fifty µl of hemocyte suspension containing ~1.2 x 10^5 cells was added to 12 mm circular coverslips in 24-well tissue culture plates containing PBS buffer (50 µl) and the cells allowed to adhere for 30 min (37 °C, 50 rpm). Integrin antibodies were used to label hemolymph-free hemocytes, and washes, occurring between blocking/permeabilization and antibody incubations, consisted of rinsing the cells in PBS (500 µl) three times. To determine the effect of CTX on the presence of lamellipodia on hemocytes and/or the frequency of filopodia per hemocyte and the frequency of anti-α_5 integrin-related fluorescent bands between interacting hemocytes, increasing concentrations of CTX were added (prior to hemocyte addition) to coverslips (50 µl), afterwhich incubations and washes were the same as before. Cells were fixed in 4% formaldehyde (v/v PBS; 500 µl) for 15 min. Since cells can contain intracellular stores of integrins (Todd *et al.*, 1984) and that integrins can associate with and influence the actin cytoskeleton (Etienne-Manneville and Hall, 2001; Hynes, 2002; Galbraith *et al.*, 2007), hemocytes were permeabilized to detect total integrin and stained for actin to detect the colocalization of integrins and the cytoskeleton. Cells were both blocked and permeabilized in 0.3% BSA (w/v) with 0.2% (v/v) Triton X-l00 in PBS (500 µl) for 30 min. Permeabilization with Triton X-100 removes the cell membrane and membrane-associated proteins leaving the cytoskeleton and possible actin-associated integrins (Galbraith *et al.*, 2007). The primary antibody [final concentration of 0.4 µg/ml in 0.3% BSA (w/v) in PBS (500 µl) for all integrin

antibodies, except β_3 (1 µg/ml)] was incubated with the cells for 2 h. Cells were then labeled with the secondary antibody [final concentration in 0.3% BSA (w/v) in PBS (500 µl): 0.1 µg/ml] for 1 h followed by actin labeling with Alexa fluor 568-labeled phalloidin [final concentration in 0.3% BSA (w/v) in PBS (500 µl): 20 µg/ml] for 1 h. Nuclei were stained with DAPI [4',6-diamidino-2-phenylindole; final concentration of 1 µg/ml in PBS (500 µl)] for 2 min and mounted onto microscope slides using 3 µl GelTol (Fisher Scientific). Control hemocytes were incubated with the secondary antibody without primary antibody labeling. Cell images under 63X magnification were analyzed with a Zeiss Axiovert 200M immunofluorescence microscope and Northern Eclipse software, while confocal images were taken with a Zeiss Axiovert 510 confocal microscope and focal plane (Z) stacks obtained. Cell types were identified with phase contrast microscopy. Images were examined and analyzed using Adobe Photoshop CS3. A minimum of 3 replicates was used, and where applicable, at least 100 hemocytes were examined per replicate for the number of filopodia and lamellipodia and at least 200 cell interactions were sampled per replicate for integrin-related fluorescent bands.

Insect injections and the level of circulating hemocytes and bacteria *in vivo*

To examine the possible involvement of integrin-like molecules in hemocyte immune responses, a given antibody type without or with *Ba. subtilis* was injected into the larval hemocoel. The use of antibodies raised against individual integrin subunits has been used to functionally inhibit whole dimers on *Ma. sexta* hemocytes and the WI38 human lung fibroblasts (Akiyama *et al.*, 1989; Wiegand *et al.*, 2000). Insects (chilled on ice for 10 min) were injected with PBS (10 µl) containing integrin antibodies without or with *Ba. subtilis* (6×10^7 cells) at the third prothoracic proleg [total volume inside insect after injection = 60 µl (Gagen and Ratcliffe, 1976)]. Insects were incubated for 20 min at 37 °C. Twenty minutes was used because it reflects the time by which hemocyte microaggregation events have occurred *in vivo* in response to bacteria (Ratcliffe and Gagen, 1977). Anti-dog IgG was used as a non-specific control for the antibody injections. Ten µl of hemolymph was then removed from the insect and the concentration of circulating bacteria and/or hemocytes determined on a hemocytometer by phase

contrast microscopy. Each insect was itself a replicate receiving individually prepared treatments with a minimum of 5 replicates per experiment.

Statistics

All data were analyzed using the 95% confidence limit overlap protocol (Sokal and Rohlf, 1969). Percentage data are recorded as the decoded mean with 95% confidence limits in 2 arcsin √p-transformation. Graphic and tabular data are presented as the mean ± standard error of the mean. An *a priori* α value of 0.05 was chosen.

RESULTS

Integrin-like molecules on hemocytes

To identify antigens recognized by the integrin antibodies, I determined the presence and molecular weights of the antigens by dot and Western blotting. Dot blots with all four integrin antibodies were positive (Fig. 3.1A,B,C,D) compared to their non-specific controls (Fig. 3.1E,F), wherein anti-α_v provided the most intense fluorescent signal. Immunoreactive integrin-like molecules (hence forth referred to as integrins) were found in the hemocyte lysate using Western blot analysis. The α_v integrin subunit antibody produced seven bands with molecular weights of 111kDa, 132kDa, 162kDa, 207kDa and three bands greater than 250kDa (Fig. 3.1G). The α_5 integrin subunit antibody produced bands with a molecular weight of 145kDa and less than 50kDa (Fig. 3.1H). The β_3 and β_1 integrin subunit antibody labeled two bands with molecular weights greater than 250kDa (Fig. 3.1I,J), and the β_3 integrin subunit antibody produced a third band 160kDa in size.

Effect of cholera toxin on adhesion structures and integrin distribution on hemocytes

Since cholera toxin stimulates hemocyte adhesion (Chapter 2), the frequency of filopodia structures, which are involved in integrin-dependent adhesion in NIH-3T3 fibroblasts (Galbraith *et al.*, 2007), was determined for both granular cells and plasmatocytes with increasing CTX levels known to affect *G. mellonella* adhesion. Actin structures extending past lamellipodial edges and terminating with a fine tip were considered filopodia. Lamellipodia were present on all plasmatocytes, and the frequency of filopodia per plasmatocyte was not significantly different ($p > 0.05$) between CTX treatment groups (Table 3.1). Not only was there no difference in granular cells with lamellipodia with increasing CTX treatment ($p > 0.05$), but there was also no change in filopodial frequency per plasmatocyte or granular cell with or without lamellipodia ($p > 0.05$). Therefore, CTX-induced hemocyte-hemocyte adhesion (Chapter 2) was independent of filopodial and lamellipodial intensity per hemocyte type, possibly

Table 3.1 Effect of whole cholera toxin on hemocyte lamellipodia and filopodia frequency

Cholera toxin (nM)	Granular cells with lamellipodia (%)[†]	Filopodia per hemocyte[†]		
		GR+[‡,§]	GR-[‡,§]	PL[†]
0.0	33.5 ± 3.0[a]	9.9 ± 1.57[b,d]	20.3 ± 1.12[c,e]	14.9 ± 0.47[d,f]
1.2	38.8 ± 3.0[a]	8.0 ± 2.17[b,d]	20.8 ± 2.53[c,e]	16.3 ± 0.48[d,f]
60.0	36.7 ± 2.9[a]	10.0 ± 1.94[b,d]	22.8 ± 2.88[c,e]	17.1 ± 0.43[d,f]
120.0	40.3 ± 3.1[a]	8.7 ± 1.13[b,d]	22.4 ± 2.50[c,e]	15.4 ± 1.04[d,f]

[†] Mean ± standard error of the mean, n=3. Values within a column with the same primary superscript were not significantly different, $p > 0.05$. Values within a row with the same secondary superscript were not significantly different, $p > 0.05$.
[‡] Granular cell subtypes with lamellipodia presented significantly fewer ($p < 0.05$) filipodia, and were categorized in a separate group.
[§] GR+ represents granular cells with lamellipodia; GR- represents granular cells without lamellipodia. PL represents plasmatocytes.

reflecting activation of cellular mechanisms proceeding and/or unrelated to actin polymerization.

The localization of the integrins on granular cells and plasmatocytes (washed free of hemolymph), differing largely between antibody type, was determined by immunofluorescence labeling. The α_v integrin subunit antibody appeared as a punctate pattern on the filopodia of both cell types (Fig. 3.2). In plasmatocytes, intracellular integrin-stores appeared in the perinuclear region, and stress fibers were heavily stained with the anti-α_v antibody in a punctate pattern. A distinct ring of fluorescence coinciding with the cell cortex (which was distinguished by the phalloidin stain) was seen around most isolated and aggregated granular cells [73% (42.2-52.0)] suggesting its presence on the surface of the cell. Bands of fluorescence between plasmatocyte-granular cell heterotypic interactions and overlapping with an intense actin stain was observed but at low frequency [5.5% (0.0-7.0)]. This intense stain was referred to as a fluorescent band due to the distinct absence of labelling in adjacent regions.

The α_5 integrin subunit antibody concentrated heavily in the perinuclear region of both plasmatocytes and granular cells, likely indicating intracellular stores, and appeared as a punctate pattern at the periphery nearing the cell cortex (Fig. 3.3). Integrin fluorescence was seen on the filopodia of both hemocyte types and on the stress fibers of the plasmatocytes, albeit the latter was less intense than with anti-α_v. The integrin subunit also intensely stained portions of hemocytes involved in cell-cell interactions overlapping with actin staining, similar to the α_v antibody but occurring at a much greater frequency [42.5% (20.3-30.2) for heterotypic interactions and 67.0% (35.8-49.0) for homotypic interactions].

The β_3 integrin subunit antibody, which more heavily labelled granular cells than plasmatocytes, appeared more evenly distributed on the granular cells with a punctate pattern appearing on the filopodia (Fig. 3.4). The plasmatocytes were intensely labelled in the perinuclear region with a punctate pattern diffusing outwards towards the cell cortex and onto the filopodia. Other plasmatocytes showed a uniform staining on lamellipodia, while no stress fibers on any plasmatocytes were labeled. No fluorescent bands occurred between interacting hemocytes.

The β_1 integrin subunit antibody, similar to the β_3 integrin subunit antibody, labelled the perinuclear region of plasmatocytes and diffused out in a punctate pattern to the cell cortex and onto the filopodia. No label appeared on the stress fibers of plasmatocytes (Fig. 3.5). The integrin stain in the granular cells was more evenly distributed throughout the cell with a less pronounced punctate pattern, and no fluorescent bands occurred between interacting hemocytes.

I determined the frequency of the fluorescent band phenotype, observed with the α_5 antibody, on hemocytes treated with increasing concentrations of CTX that induce microaggregates *in vitro* and nodule-like structures *in vivo* (Chapter 2). Band frequency was determined by immediate direct observation, since negative and positive fluorescent band phenotypes were easily distinguishable (see Fig. 3.5). Overlapping hemocytes displayed two phenotypes, those with an intense phalloidin stain on the overlapping portion of the membrane and those without the stain. The latter could indicate a weaker or non-existent interaction between hemocytes, implying that the presence of the intense phalloidin stain is indicative of biochemically interacting hemocytes. Interacting hemocytes with an α_5 fluorescent band displayed also the intense phalloidin stain, however, the intense phalloidin stain was not indicative of an α_5 fluorescent band suggesting bleed-through of the phalloidin signal had no effect on the integrin fluorescence pattern. Without CTX, the band frequency was similar ($p > 0.05$) between homotypic hemocyte interactions and significantly greater ($p < 0.05$) than the band frequency in granular cell-plasmatocyte interactions (Table 3.2). Band frequency significantly increased between interacting plasmatocytes and granular cells with increasing CTX treatment by 30% points ($p < 0.05$) displaying levels similar ($p > 0.05$) to those seen in homotypic hemocyte interactions, which may indicate increasing plasmatocyte-granular cell interactions. Band frequency between similar interacting hemocyte types remained the same ($p > 0.05$) regardless of CTX treatment. Band frequency between plasmatocytes and granular cells displayed high correlation coefficients with *in vitro* parameters examined in Chapter 2, such as total individually attached hemocytes ($R = -0.97$; $p > 0.05$) and total sum of aggregated hemocytes (adhered and non-adhered to glass; $R = 0.99$; $p > 0.05$), but not total aggregated

Table 3.2 Effect of whole cholera toxin on the frequency of the α_5 integrin subunit-dependent fluorescent band between interacting hemocytes

Whole cholera toxin (nM)	Percentage of hemocyte interactions with fluorescent bands[†]		
	Granular cell - Granular cell	Plasmatocyte - Plasmatocyte	Granular cell - Plasmatocyte
0	$63.7 \pm 3.0^{a,e}$	$70.2 \pm 2.9^{b,e}$	$42.5 \pm 2.7^{c,f}$
1.2	$74.4 \pm 2.3^{a,g}$	$74.5 \pm 2.3^{b,g}$	$74.9 \pm 1.8^{d,g}$
120	$76.1 \pm 2.0^{a,h}$	$70.4 \pm 2.6^{b,h}$	$69.0 \pm 2.0^{d,h}$

[†] Mean ± standard error of the mean, n=3. Values within a column with the same primary superscript were not significantly different, $p > 0.05$. Values within a row with the same secondary superscript were not significantly different, $p > 0.05$.

hemocytes attached to glass ($R = 0.77$; $p > 0.05$). Furthermore, band frequency between plasmatocytes and granular cells displayed high correlation coefficients with the *in vivo* parameters, including circulating hemocytes in the hemocoel ($R = 0.96$; $p > 0.05$), *Ba. subtilis* removal ($R = -0.87$; $p > 0.05$), and nodule formation in the insect ($R = 0.97$; $p > 0.05$), after correcting for tissue dilution effects *in vivo*. These correlations were not statistically significant due to the low number of treatment groups, however, the recurring patterns between all parameters suggest biological significance. Because the frequency of the fluorescent band increased with CTX treatment and CTX induced a nodulation-like response, the hemocyte organization and structure of the *in vitro* hemocyte microaggregates, and the localization of the α_5 integrin subunit therein, was examined with confocal microscopy (Fig. 3.6). Transverse sections (Fig. 3.6A, B, C, D) at the hemocyte-substratum interface displayed a monolayer of plasmatocytes, whereas higher sections demonstrated a cluster of spherical granular cells. A coronal section (Fig. 3.6E, F) of the *in vitro* microaggregate more clearly illustrated the arrangement of granular cells and plasmatocytes, where a cluster of granular cells sat atop a monolayer of plasmatocytes. *In vitro* microaggregates were labeled also with anti-α_5 integrin subunit, but no fluorescent band pattern was seen between interacting hemocytes, which may be the result of the compactness of the *in vitro* microaggregate. The structure of *in vitro* microaggregates formed with (Fig. 3.6A, C, E) or without (Fig. 3.6B, D, F) CTX was the same, but the size of *in vitro* microaggregates formed with CTX was larger, which corresponded to larger clusters of granular cells atop more plasmatocytes.

Effect of integrin antibodies on total hemocyte counts and bacterial removal

Hemocytes in the hemocoel can enter and exit the hemolymph by modifying their adhesiveness for surrounding tissues (Wigglesworth, 1956; Jones, 1962; Gagen and Ratcliffe, 1976) rapidly changing total hemocyte counts faster than is possible for hemocytic mitosis (Mangalika *et al.*, 2009; Markus *et al.*, 2009). That all the integrin antibodies labeled both plasmatocytes and granular cells and the anti-α_5 fluorescent band frequency increased with CTX treatment, I assessed whether the antibodies could modulate hemocyte activity and/or the process of bacterial removal from the insect

hemocoel. Injection of anti-dog IgG as a non-specific control did not affect either total hemocyte counts or bacterial removal from the hemolymph (Fig. 3.7A). Neither anti-α_v (Fig. 3.7B) nor anti-β_3 (Fig. 3.7C) antibodies only influenced total hemocyte counts at the antibody levels used, anti-β_3 being the inexplicable exception lowering total hemocyte counts at 500 pg/ml. Furthermore, neither antibody influenced *Ba. subtilis* removal. Total hemocyte counts in larvae with anti-α_5 antibodies only did not change over antibody concentration (Fig. 3.7D), whereas larvae with anti-β_1 antibody only exhibited a concentration-dependent increase in total hemocyte counts ($R = 0.74$, $p > 0.05$; Fig. 3.7E). The latter two antibodies increased bacterial levels with increasing antibody concentration, indicating increasing impairment of bacterial removal, anti-β_1 ($R = 0.94$, $p < 0.05$) being more effective than anti-α_5 ($R = 0.65$, $p > 0.05$). Injections of anti-α_5 antibody with bacteria did not impair hemocyte decline after the initial drop that occurred during the incubation period, whereas injections of anti-β_1 with bacteria continued hemocytopenia.

DISCUSSION

In Chapter 2, I established that CTX-induced *in vitro* microaggregation could be inhibited by an RGD peptide impliying the involvement of integrins. Hemocyte-hemocyte interactions are a crucial part of the nodulation and encapsulation responses, the involvement of integrins having been alluded to in polystyrene-stimulated *Ma. sexta* hemocyte-hemocyte adhesion (Nardi *et al.*, 2005; Zhuang *et al.*, 2007b). The basic β subunits of integrins can be found in the more primitive metazoans, including coral (P. Cnidaria, *Acropora millepora*), sponges (P. Porifera, *Ophlitaspongia tennis*; Brower *et al.*, 1997), and nematodes (*Caenorhabditis elegans*; Gettner *et al.*, 1995), numerous insect and crustacean arthropod species (Brower *et al.*, 1995; Holmblad *et al.*, 1997; Levin *et al.*, 2005) and humans (Hynes, 2002). The integrin α subunits from insects and humans exhibit conserved primary structures (Zhuang *et al.*, 2008); in the case of nematodes and crustaceans, the tertiary structure of the β subunits possess similar folding (Holmblad *et al.*, 1997). The integrin subunits herein characterized by human polyclonal antibodies [all of which participate in mammalian integrin dimers recognizing the RGD motif (Hynes, 2002)] are on hemocytes from gastropods (Plows *et al.*, 2006), bivalves (Terahara *et al.*, 2006), and insectan dipterans (Foukas *et al.*, 1998), and lepidopterans (Lavine and Strand, 2003; Levin *et al.*, 2005). The homology described justifies using human integrin antibodies to examine the involvement of integrins in *G. mellonella* hemocyte immune responses.

That integrins are associated with the systemic immune response of *G. mellonella* is based on the following: 1) integrin antibodies recognize molecules in plasma-free, whole hemocyte lysates, 2) integrin antibodies label the hemocytes, the cellular arms of the innate immune response, 3) the anti-α_5 antibody labels the junction between interacting hemocytes, this band increasing with CTX treatment, and 4) antibodies anti-α_5 and anti-β_1 inhibit bacterial removal from the hemolymph. The implication is that integrins influence hemocyte-microbe interactions possibly by stimulating adhesion and by extension phagocytosis or nodulation.

Insect hemocyte-hemocyte interactions are important in isolating large numbers of small microbes (nodulation) and large microorganisms (encapsulation) in the

hemolymph (Ratcliffe and Gagen, 1977; Schmit and Ratcliffe, 1977). The process of resisting infection in *G. mellonella* begins with granular cell stimulation (Ratcliffe and Gagen, 1977), followed by plasmatocytes settling onto tissues (Chain and Anderson, 1982; Scapigliati and Mazzini, 1994). Nodulation then proceeds when granular cells interact to form microaggregates, containing also a few plasmatocytes, incorporating and isolating the microbes (Ratcliffe and Gagen, 1977; Stanley and Miller, 2006). Microaggregates, which settle onto and adhere to surrounding tissues, grow larger as more granular cells adhere to its outer surface. The addition of granular cells to the microaggregates eventually ceases, but microaggregate growth continues as plasmatocytes adhere to the multicellular structures ultimately forming the nodule (Ratcliffe and Gagen, 1977). That α_5 and β_1 integrins appear to participate in *G. mellonella* hemocytic responses has a counterpart in *Ma. sexta* in that β integrin subunit monoclonal antibodies and dsRNA against α subunits will inhibit encapsulation of Sephadex beads (Wiegand *et al.*, 2000; Zhuang *et al*., 2008). Inhibiting *Ma. sexta* hemocyte integrin-associated surface proteins, tetraspanin and neuroglian, will inhibit also the encapsulation response (Zhuang *et al*., 2007a; Zhuang *et al.*, 2007b), reflecting their involvement in mediating *in vitro* homotypic and heterotypic interactions between plasmatocytes and granular cells (Nardi *et al.*, 2006, Zhuang *et al.*, 2007b). That capsules and nodules are closely related immune structures (Ratcliffe and Gagen, 1977; Schmit and Ratcliffe, 1977) implies similar involvement of integrins in nodulation. The α_5 antibody labeled the junction between interacting hemocytes, the typical location for a molecule involved in cell-cell interactions (Hynes, 2002). The fluorescent band between interacting *G. mellonella* larval hemocytes are more predominant in homotypic interactions than between heterotypic interactions. When plasmatocytes and granular cells are treated with increasing concentrations of CTX, inducing RGD-dependent hemocyte microaggregations *in vitro* and nodule-like structures *in vivo* (see Chapter 2), the frequency of fluorescent α_5 bands in heterotypic interactions increases to levels observed in homotypic interactions. This suggests that the effect of CTX on increasing *in vitro* microaggregation is mediated in part by the α_5 integrin, and that adhesion between granular cells and plasmatocytes contributes significantly to the growth of the nodule.

In chapter 2, we established that the effects of high concentrations of CTX may be mediated by the cholera toxin B-subunit (CTB), whereas the cholera toxin A-subunit (CTA) is likely mediating effects at the lower concentrations. AB_5 proteins, such as CTX and pertussis toxin (PTX), can induce hemagglutination of mammalian blood cells (Sugii, 1987, Schmidt and Schmidt, 1989; Capodici *et al.*, 1998). The frequency of the α_5 band in heterotypic hemocyte interactions is similar between low and high CTX treatments, and this suggests that both CTA and CTB might induce *in vitro* hemocyte microaggregation by way of similar integrins. GM1 glanglioside/integrin receptor complexes on neuroblastoma cell lines bind CTB (Wu *et al.*, 2007) and anti-β integrins on *Ma. sexta* hemocytes colocalize to lipid rafts that bind also CTB (and thus GM1 gangliosides; Nardi *et al.*, 2005). That the frequency of α_5 integrin bands increases with higher CTX concentration treatment, indicative of the CTB effect, may represent GM1/integrin-associated lipid raft signaling culminating in hemocyte-hemocyte adhesion. Possibly CTB may direct this reaction by interacting with integrins directly, as reported for the B-subunit of PTX which binds to integrins on human macrophages (Saukkonen *et al.*, 1992; Wout *et al.*, 1992). In the case of the lower CTX concentration treatment, $G\alpha_s$ proteins are likely inducing an integrin-dependent microaggregation response; measuring levels of intracellular cAMP will confirm this. Also, that Gα subunits can direct integrin-related hemocyte-hemocyte adhesion interactions is not unexpected since $G\alpha_{13}$ subunits associate with the cytoplasmic tails of β_3 integrins in mouse platelets to mediate cell spreading (Gong *et al.*, 2010). However, whether the presence of integrins between CTX-mediated hemocyte-hemocyte interactions is the result of activated Gα subunits or the indirect consequence of increased hemocyte-hemocyte adhesion remains to be determined. Loading the α_5 antibody osmotically into hemocytes or inhibiting integrin expression by way of RNAi will be considered. The α_5 antibody also colocalized with an intense actin stain in homotypic and heterotypic hemocyte interactions, which may imply the formation of focal adhesions. Such confirmation requires staining for focal adhesion proteins, such as vinculin or paxilin.

The structure of the *in vitro* hemocyte microaggregate described herein resembles those in the larvae of three saturniids (Walters, 1970) and offers some insight into early nodule formation *in vivo*. In my study, the *in vitro* microaggregate displays a cluster of

granular cells anchored to the substratum by a monolayer of plasmatocytes. *In vivo*, the microaggregate is predominantly composed of granular cells with some plasmatocytes (Ratcliffe and Gagen, 1977). In other lepidopterans, these plasmatocytes appear to help anchor the *in vitro* microaggregate to the substratum while others migrate away from the *in vitro* microaggregate forming a rosetting pattern around the hemocyte coacervate (Davies and Preston, 1985). Plasmatocyte subtypes can attach to a substratum and act as focal points for granular cell coacervate attachment (Dean *et al.*, 2004a; Nardi, *et al.* 2006; Zhuang *et al.*, 2007a), and since plasmatocytes are the first hemocyte type to settle on and adhere to tissues after microbial stimulation (Chain and Anderson, 1982; Scapigliati and Mazzini, 1994) this may provide evidence for their importance in adhering microaggregates to surrounding tissues during nodulation, similar to what I see *in vitro*. Plasmatocytes rosetting around the *in vitro* microaggregate (Davies and Preston, 1985; chapter 2 of this thesis) may represent hemocytes responsible for further anchoring *in vivo* nodules as they continue to increase in size (as more granular cells adhere to the microaggregate). This would explain the relationship between increasing α_5 integrin-related interactions between plasmatocytes and granular cells and increasing microaggregation with increasing CTX concentration.

Filopodia on *Bombyx mori* granular cells elongate in response to foreign material and stimulate phagocytosis (Wago, 1981; 1983a; 1983b; 1984). Disrupting actin polymerization inhibits hemocyte attachment, phagocytosis, and encapsulation (Wago, 1981; 1984; Li, *et al.*, 2009). Lamellipodia are associated with cell spreading in 3T3 fibroblasts (Price *et al.*, 1998), inhibiting hemocyte spreading can disrupt encapsulation responses against parasitoid wasp eggs as in *Pse. separata* (Suzuki *et al.*, 2008). That CTX has no effect on *G. mellonella* filopodial frequency on plasmatocytes or granular cells, the latter which consisted of a subtype with lamellipodia that is also unaffected by CTX, suggests that RGD-dependent *in vitro* hemocyte microaggregation does not depend on a subtype of granular cells with lamellipodia or filopodial frequency on either plasmatocytes or granular cells. However, increased microaggregation may reflect recruitment and occupancy of integrins on filopodial and lamellipodial structures or elongation of integrin-containing filopodia, as seen in 3T3 fibroblasts (Partridge *et al.*, 2006; Guillou *et al.*, 2008).

Hemocyte counts and bacterial removal from the hemolymph of larval *G. mellonella* varies with the type and concentration of injected integrin subunit antibody. Although α_v and β_3 subunit antibodies had no significant effect on these parameters, it does not mean the subunits are not on the hemocytes since immunofluorescent microscopy established subunits on the granular cells and plasmatocytes. Both hemocyte types have also α_5 and β_1 subunits which impact on non-self reactions albeit in inexplicably different ways. The antibodies to both subunits caused an antibody concentration-dependent increase in *Ba. subtilis* levels, the anti-β_1 subunit antibody being more effective than the anti-α_5 antibody. It is unlikely that these results represent non-specific binding to hemocyte-modulating plasma proteins or inhibition of bacterial adhesion to insect tissues (including hemocytes) because neither hemocyte nor bacterial levels were affected with anti-dog IgG, -α_v or -β_3 subunit antibodies and the levels were disproportionate between α_5 and β_1 antibodies, implying that the latter effects were hemocyte-dependent. Whether the α_5 and β_1 antibodies recognize similar integrins is unknown, but the human $\alpha_5\beta_1$ integrin is known to mediate adhesion, migration, and bactericidal activities in neutrophils (Simms and D'amico, 1997; Loike *et al.*, 1999) and stimulate phagocytosis of fibronectin-opsonized particles by both neutrophils and macrophages (Brown and Goodwin, 1988). However, although the α subunit antibodies bound hemocyte molecules with molecular weights closely resembling the weights of their human counterparts (α_v ~125 kDa; α_5 ~140 kDa; Pytela *et al.*, 1985; Roman *et al.*, 1989), the β subunit antibodies did not. How the α_5 and β_1 antibodies disrupt bacterial removal is unknown, but that the α_5 integrin is involved in CTX-induced *in vitro* hemocyte microaggregation suggests the α_5 antibody prevents hemocyte interactions and ultimately nodulation.

The presence of intracellular stores of integrins allows their release from neutrophil granules (degranulation) and transfer to the plasma membrane, likely by granule membrane fusion with the plasma membrane (Todd *et al.*, 1984), afterwhich adhesion to foreign surfaces typically occurs (Wright and Gallin, 1979; Bockenstedt and Goetzl, 1980). The presence of integrin stores left in plasmatocytes and granular cells adhering to coverslips may represent sub-maximal release of integrin stores, much like medfly hemocytes which use different receptors to adhere to biotic and abiotic surfaces

(Lamprou *et al.*, 2007). Different integrins can be released from neutrophil granules under separate stimuli (Todd *et al.*, 1984), which may also explain leftover hemocyte integrin stores and why both β_3 and α_v antibodies did not participate in bacterial removal, despite the latter's presence on coverslip-bound granular cell surfaces. The β_3 and α_v integrin molecules are may be used for different adhesion events.

Herein, I have shown that a human α_5 integrin antibody is capable of inhibiting bacterial removal from the insect hemocoel and labeling the junction between interacting hemocytes producing a band. The frequency of this band increases in heterotypic hemocyte interactions with increasing CTX concentration, strongly supporting the involvment of integrins in nodulation since CTX is responsible for inducing *in vitro* hemocyte microaggregation. Furthermore, the cell agglutinating effect of high concentrations of CTX, achieved by CTB, is integrin-related. Also, that the lower CTX concentrations, likely induced by CTA, mediate also *in vitro* microaggregation and stimulate α_5 labeling between plasmatocytes and granular cells suggests potential mediation of integrin-dependent hemocyte microaggregation by $G\alpha_s$ proteins.

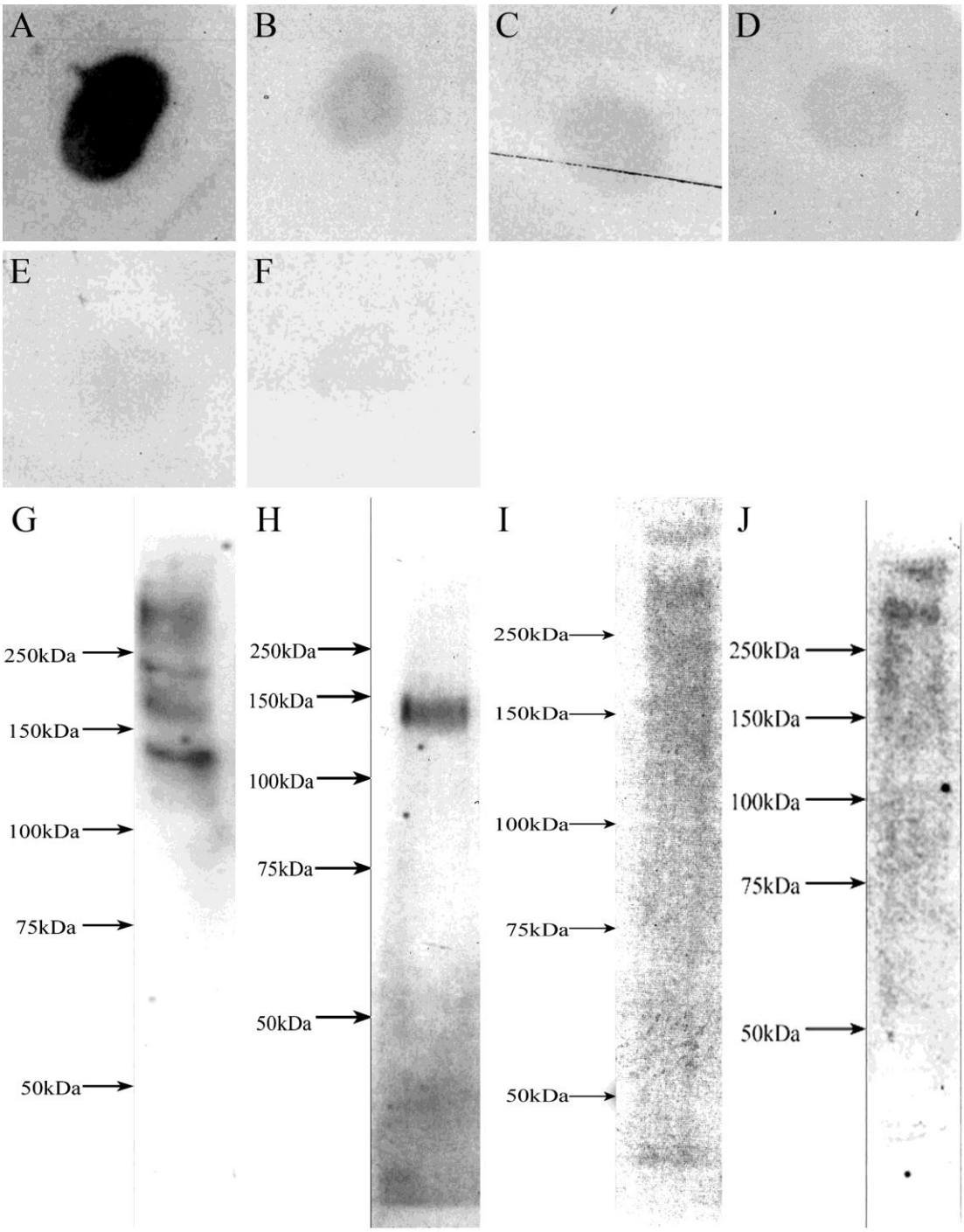

Figure 3.1. Immunoluminescent dot and Western blots on whole hemocyte lysates with the integrin antibodies.

Dot blots of hemocyte lysates with positive results with the αv (A), α5 (B), β3 (C), and β1 (D) integrin subunit antibodies are shown. Non-specific controls included incubating the membrane with the secondary antibody without primary antibody labeling. The non-specific control for β1, β3, and αv is shown in (E) and α5 in (F). Proteins in the hemocyte lysate were separated by SDS-PAGE before being transferred to nitrocellulose and the membranes were probed with the αv (G), α5 (H), β3 (I), and β1 (J) integrin subunit antibodies.

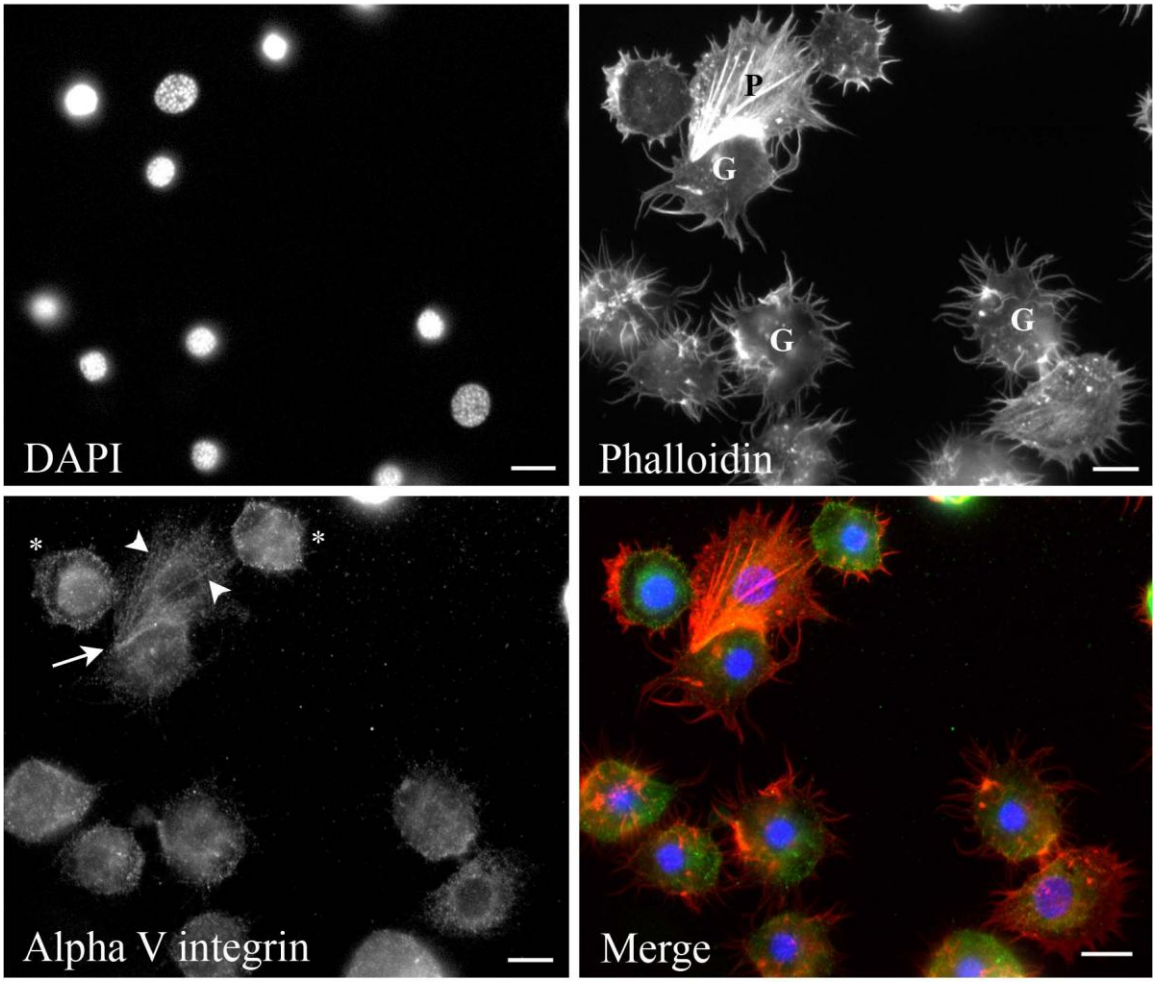

Figure 3.2. The αv integrin subunit antibody stains plasmatocytes and granular cells.

The integrin staining appears in a punctate pattern in the filopodia of both cell types. The stress fibers of the plasmatocytes are heavily stained (arrowheads), and a distinct ring of fluorescence coinciding with the cell cortex can be seen around most granular cells (asterisk). Fluorescence between interacting hemocytes (arrow) and overlapping with an intense actin stain was observed but occurred in low frequency. The letters seen in the phalloidin labelled image denote the hemocyte type; 'G' for granular cell and 'P' for plasmatocyte. The scale bar represents 10 μm.

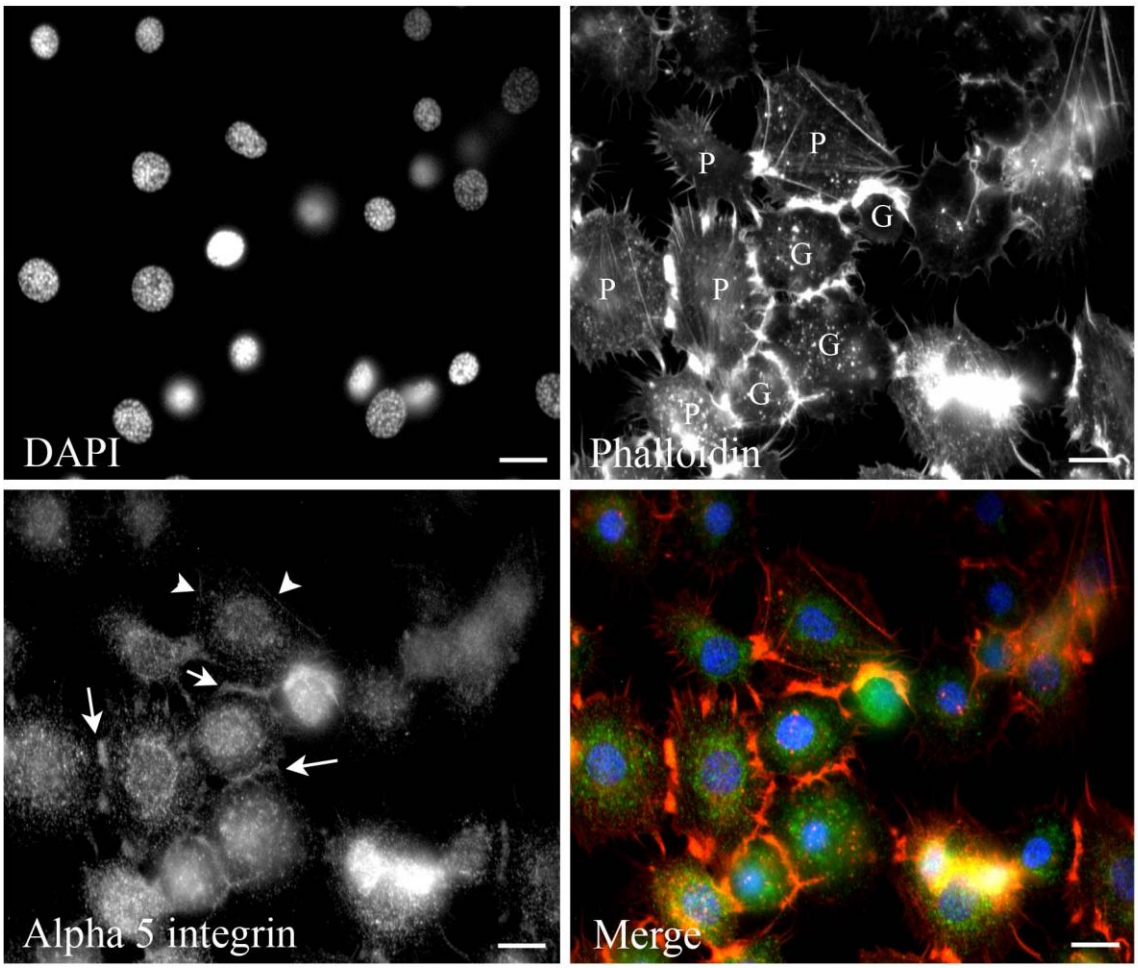

Figure 3.3. The α5 integrin subunit antibody stains plasmatocytes and granular cells.

The integrin staining concentrates heavily around the nucleus and appears as a punctate pattern at the periphery of both cell types. Fluorescence can be seen on the filopodia of both hemocytes and on the stress fibers (arrowheads) of the plasmatocytes. Between interacting cells, coinciding with an intense actin stain, are fluorescent bands (arrows). The letters seen in the phalloidin labelled image denote the hemocyte type; 'G' for granular cell and 'P' for plasmatocyte. The scale bar represents 10 μm.

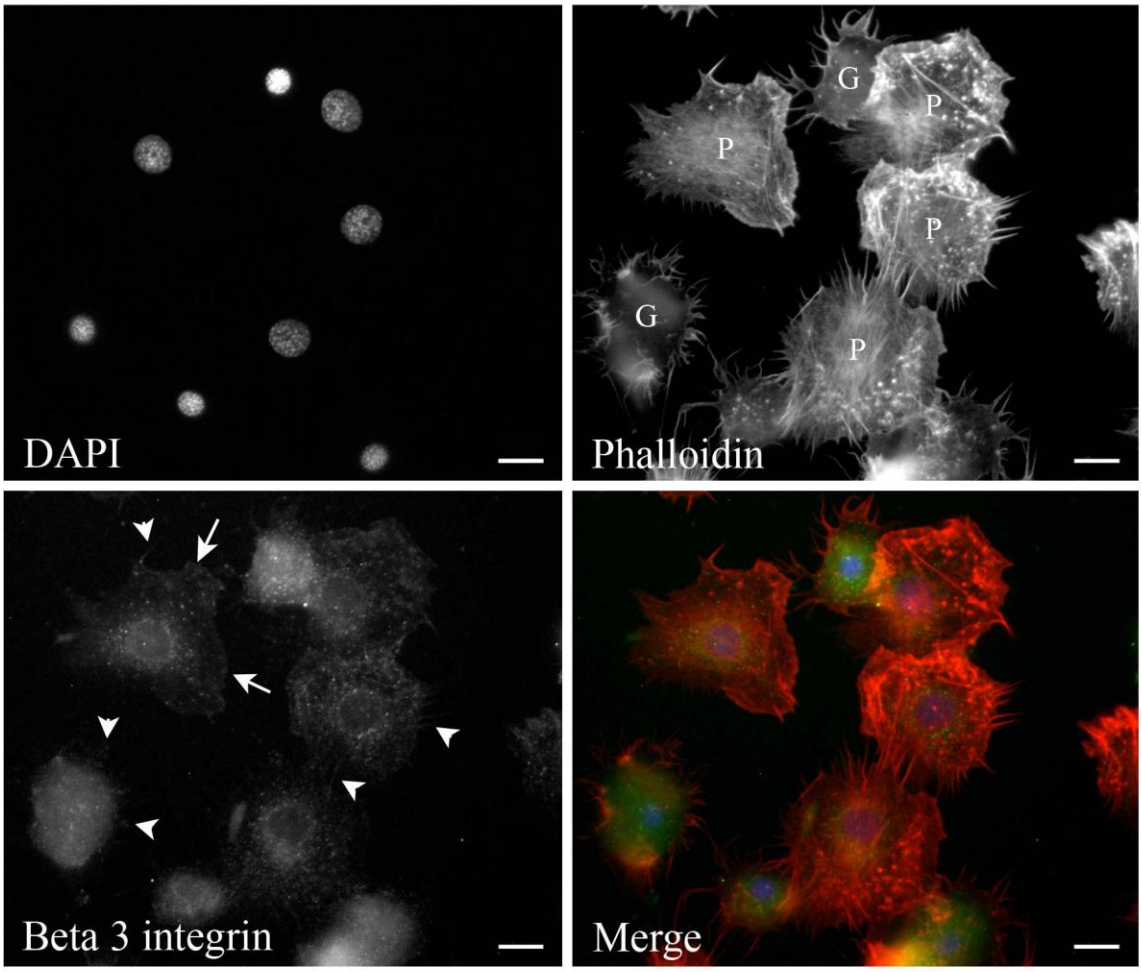

Figure 3.4. The β3 integrin subunit antibody stains plasmatocytes and granular cells.

Integrin staining appears more evenly distributed around the granular cells with a punctate pattern extending into the filopodia (arrowheads). The plasmatocytes were intensely labelled in the perinuclear region with a punctate pattern diffusing outwards towards the cell cortex and onto the filopodia (arrowheads) with other cells showing a uniform staining on lamellipodia (arrows). The letters seen in the phalloidin labelled image denote the hemocyte type; 'G' for granular cell and 'P' for plasmatocyte. The scale bar represents 10 μm.

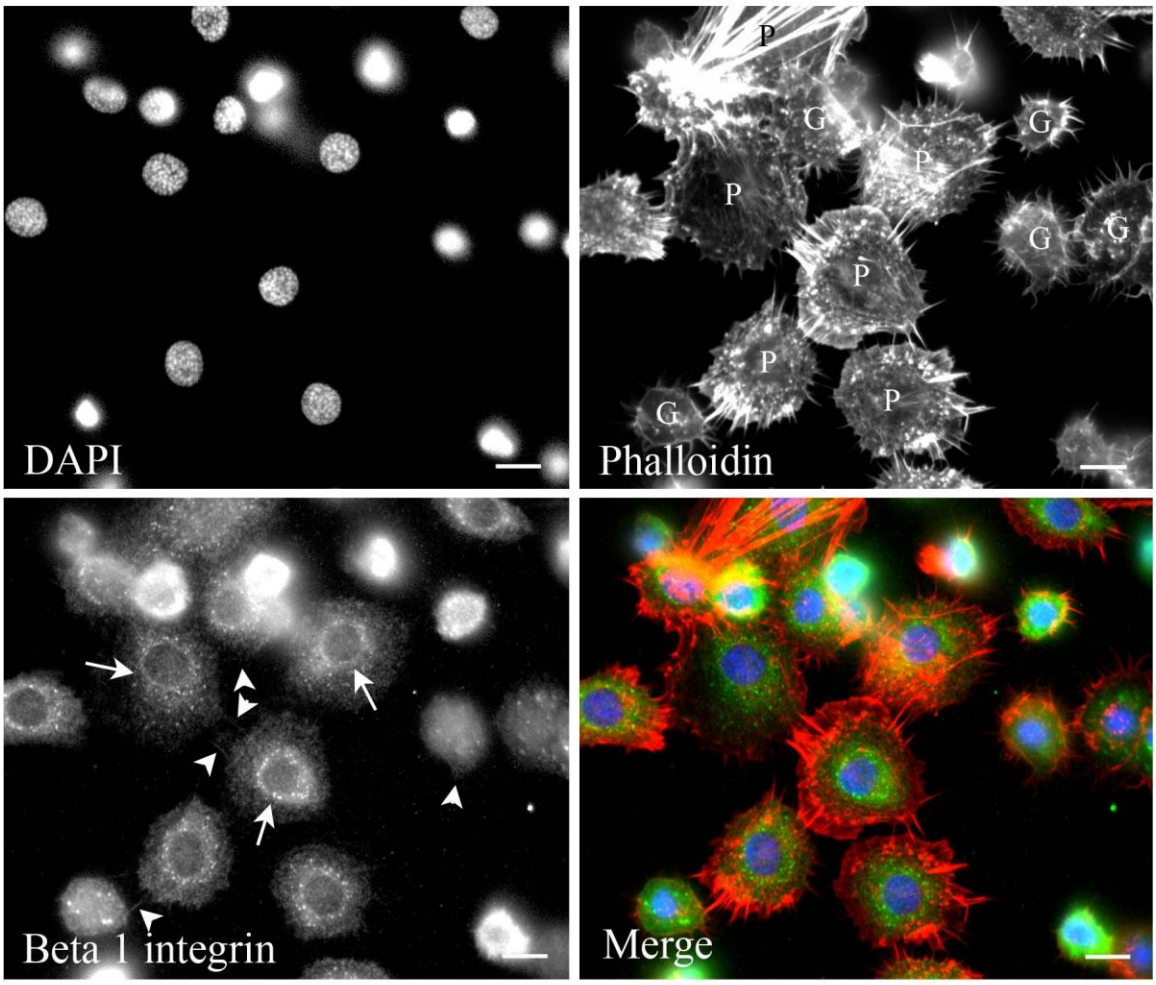

Figure 3.5. The β1 integrin subunit antibody stains plasmatocytes and granular cells.

Integrin staining appears concentrated in the perinuclear region (arrows) of plasmatocytes and diffuses out in a punctate pattern to the periphery of the cell onto the filopodia (arrowheads). The integrin staining in the granular cells is more evenly distributed throughout the cell with a less pronounced punctate pattern. The stain can also be seen on the granular cell filopodia (arrowheads). The letters seen in the phalloidin labelled image denote the hemocyte type; 'G' for granular cell and 'P' for plasmatocyte. The scale bar represents 10 μm.

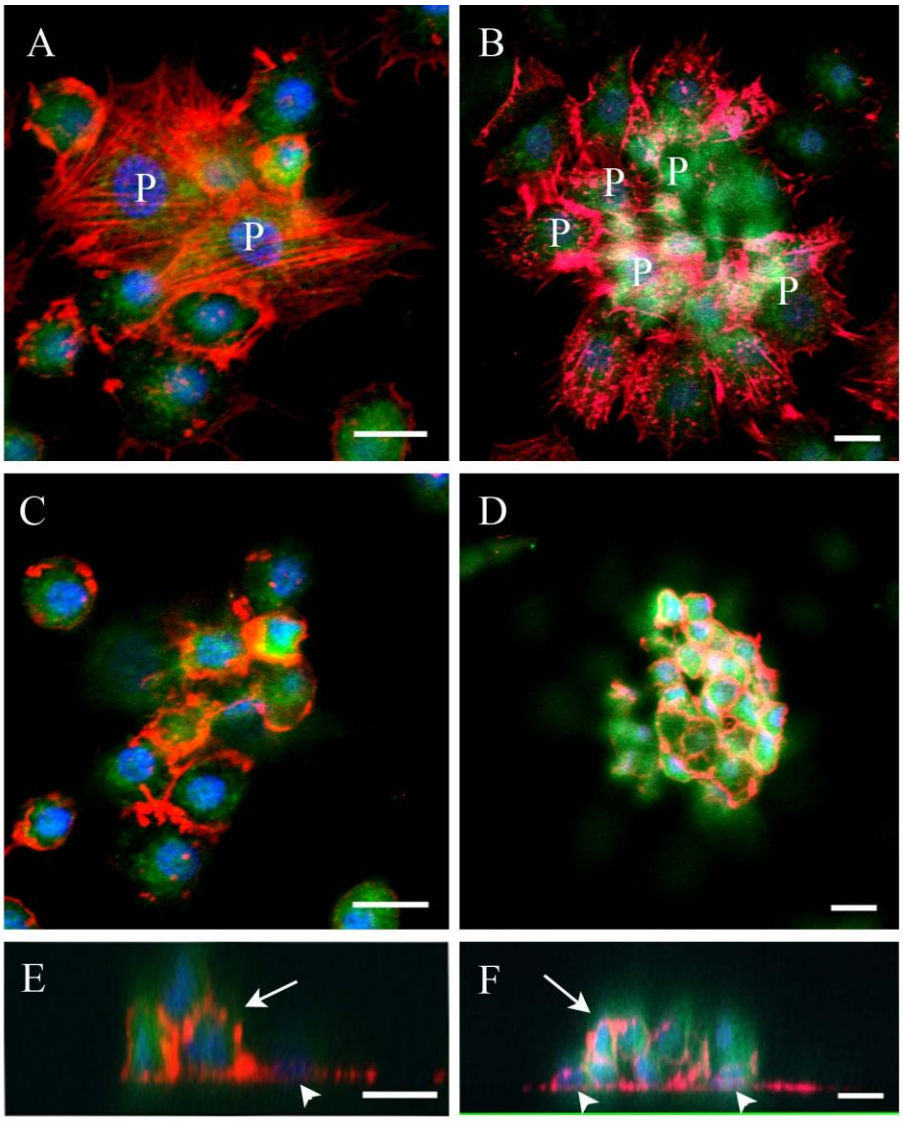

Figure 3.6. Structure of an *in vitro* hemocyte microaggregate.
Confocal images of *in vitro* hemocyte microaggregates formed with 0nM (A,C,E) and 1.2 nM (B,D,F) whole cholera toxin with hemocytes labeled with anti-α5 integrin subunit (green), actin (red), and DAPI (blue). Similar *in vitro* microaggregate structures were seen with 120 nM whole cholera toxin but are not shown. Note the similarities between *in vitro* microaggregates induced with or without whole cholera toxin. Images A-D represent transverse sections, where images A and B occur at the hemocyte-substratum interface depicting a monolayer of plasmatocytes (P), and images C and D occur above the hemocyte-substratum interface and depict a cluster of granular cells sitting atop the monolayer of plasmatocytes. Images E and F represent a coronal section and display the depth of the *in vitro* microaggregate. The arrowheads indicate the spreading plasmatocytes on the substratum, and the arrows reveal the granular cell cluster on top of the plasmatocytes.

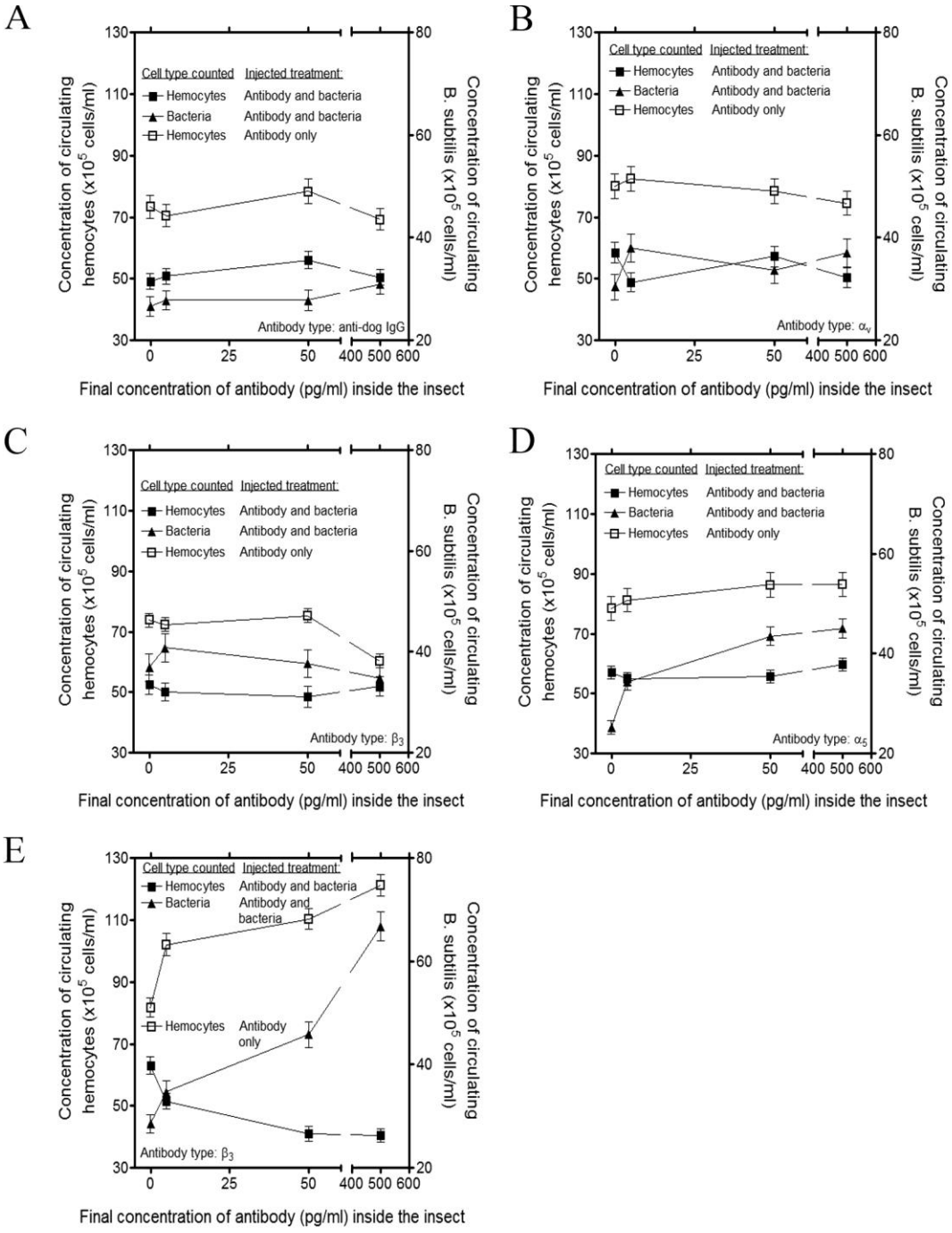

Figure 3.7. Intra-hemocoelic injections of various integrin antibodies and their effect on total hemocyte counts and the ability of hemocytes to remove bacteria.
Effects of increasing concentrations of anti-dog IgG (non-specific control; A), anti-αv integrin subunit (B), anti-β3 integrin subunit (C), anti-α5 integrin subunit (D), and anti-β1 integrin antibody (E) on the concentration of circulating hemocytes without or with bacteria and the ability of hemocytes to remove bacteria.

CONCLUSIONS

Insect blood cell nodulation is an event resulting from immune recognition and includes hemocyte-microbe and hemocyte-hemocyte interactions. This immune response requires both granular cell adhesion to the microbe and both homotypic and heterotypic granular cell and plasmatocyte interactions.

Herein, I describe the involvement of $G\alpha_s$ proteins, stimulated by cholera toxin, in both cell-substratum and cell-cell interactions. Cholera toxin, used as a G protein modulator, had a bimodal effect on hemocyte adhesion *in vitro* and *in vivo*. Lower concentrations of cholera toxin, likely mediated by its A-subunit, induced *in vitro* hemocyte microaggregates and nodule-like structures *in vivo*, which was similar in magnitude to higher levels of whole cholera toxin, but with reduced hemocyte-substratum adhesion. That higher levels of whole cholera toxin, and corresponding levels of its B-subunit, stimulated similar levels of hemocyte-hemocyte adhesion, suggests the B-subunit mediates the effects of whole cholera toxin at higher concentrations. Cholera toxin-induced *in vitro* hemocyte microaggregation was prevented by the addition of RGD peptides. In keeping with its potential involvement in nodulation, injected cholera toxin increased the removal rate of bacteria from the hemocoel of the larvae.

Furthermore, human integrin antibodies labeled integrins on the surface of the hemocytes. The human antibodies anti-β_1 and -α_5 integrin subunits were capable of inhibiting bacterial removal from the insect hemocoel, and the α_5 integrin antibody labeled the junction between interacting plasmatocytes and granular cells producing a band, the band frequency increasing with cholera toxin levels. This provides a possible link between integrins and the $G\alpha_s$-induced *in vitro* microaggregates and the nodule-like structures *in vivo*.

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