Grasshopper hemagglutinin : immunochemical localization in hemocytes and confirmation of non-opsonic properties

by Roger Steven Bradley

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry
Montana State University

Abstract:
Hemagglutinins are lectins: proteins that recognize and bind to specific carbohydrate residues on cell surfaces. Soluble hemagglutinins have been demonstrated in the hemo-lymph of a wide variety of invertebrates. In light of their carbohydrate recognitory capabilities, agglutinins have been implicated in invertebrate defense responses against pathogens, and nonself or foreign tissues and cells. Consistent with this possiblity, agglutinins are known to be associated with the circulating hemocytes in many invertebrate species, including molluscs and insects. Opsonic capacity has been demonstrated for molluscan agglutinins, however, no solid evidence for opsonic activity has been found among the insect agglutinins studied.

The objectives of this study are to localize hemagglutinin to grasshopper hemocytes using immunochemical techniques, and to investigate the in vitro opsonic properties of grasshopper agglutinin. Results of immunocytochemical staining of monolayer hemocytes reveal the presence of agglutinin in the adult hemocytes of both Melanoplus differential is and sanguinipes. Granular cells only, stain for the presence of agglutinin, the phagocytic plas-matocytes do not stain.. The hemocytes bind asialo human erythrocytes, Nosema locustae spores, and Bacillus thuringiensis bacteria. Only the resetting, of asialo human erythrocytes can be inhibited by the addition of agglutinin-specific carbohydrates. No opsonic activity is found when asialo human erythrocytes or thuringiensis is incubated in either grasshopper serum or affinity purified hemagglutinin prior to over layering hemocytes. While a potential opsonic effect is seen for Nosema spores incubated in grasshopper serum, no opsonic activity is demonstrated for Nosema incubated in purified hemagglutinin prior to over layering hemocytes. Neither increased immunofluorescence nor increased phagocytosis was observed when hemocytes, in vitro, were incubated with purified agglutinin. Phagocytosis of Nosema spores is not inhibited by the addition of agglutinin-specific monoclonal antibody to hemocytes. These results indicate that although grasshopper hemagglutinin is demonstrated in a percentage of grasshopper hemocytes, it does not appear to have an in vitro role in the recognition of foreign particles by hemocytes. Potential physiological roles of agglutinins in grasshoppers have not previously been described. Results reported here are consistent with the non-opsonic character and hemocytic location of other described insectan agglutinins.

GRASSHOPPER HEMAGGLUTININ: IMMUNOCHEMICAL LOCALIZATION IN HEMOCYTES AND CONFIRMATION OF NON-OPSONIC PROPERTIES

by

Roger Steven Bradley

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Biochemistry

MONTANA STATE UNIVERSITY
Bozeman, Montana

August 1987

APPROVAL

of a thesis submitted by

Roger Steven Bradley

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

August 20 1987
Date

Kenneth D Hopper
Chairperson, Graduate Committee

Approved for the Major Department

August 21, 1987
Date

Edwin H Abbott
Head, Major Department

Approved for the College of Graduate Studies

8-25-87
Date

Graduate Dean

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made.

Permission for extensive quotation from or reproduction of this thesis may be granted by my major professor, or in his absence, by the Dean of Libraries when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature Roger Steven Bradley

Date August 21, 1987

ACKNOWLEDGMENTS

I would like to express my gratitude to my research advisor, Dr. Kenneth D. Hapner, for his guidance during the course of this research project.

I also wish to thank the following persons from Montana State University who generously offered advice and assistance during the course of this study.

Dr. Larry L. Jackson, Chemistry Dept.

Dr. John E. Henry, USDA Rangeland Insect Lab.

Dr. Brad Stiles, Chemistry Dept.

Sharon J. Hapner, Biology Dept.

Gwendy S. Stuart, Chemistry Dept.

Zelda B. Taylor, Chemistry Dept.

TABLE OF CONTENTS

TABLE OF CONTENTS--Continued

TABLE OF CONTENTS--Continued

LIST OF FIGURES

LIST OF FIGURES--Continued

LIST OF FIGURES--Continued

ABSTRACT

Hemagglutinins are lectins: proteins that recognize and bind to specific carbohydrate residues on cell surfaces. Soluble hemagglutinins have been demonstrated in the hemolymph of a wide variety of invertebrates. In light of their carbohydrate recognitory capabilities, agglutinins have been implicated in invertebrate defense responses against pathogens, and nonself or foreign tissues and cells. Consistent with this possiblity, agglutinins are known to be associated with the circulating hemocytes in many invertebrate species, including molluscs and insects. Opsonic capacity has been demonstrated for molluscan agglutinins, however, no solid evidence for opsonic activity has been found among the insect agglutinins studied.

The objectives of this study are to localize hemagglutinin to grasshopper hemocytes using immunochemical techniques, and to investigate the *in vitro* opsonic properties of grasshopper agglutinin. Results of immunocytochemical staining of monolayer hemocytes reveal the presence of agglutinin in the adult hemocytes of both *Melanoplus differentialis* and *M. sanguinipes*. Granular cells only, stain for the presence of agglutinin, the phagocytic plasmatocytes do not stain. The hemocytes bind asialo human erythrocytes, *Nosema locustae* spores, and *Bacillus thuringiensis* bacteria. Only the rosetting of asialo human erythrocytes can be inhibited by the addition of agglutinin-specific carbohydrates. No opsonic activity is found when asialo human erythrocytes or *B. thuringiensis* is incubated in either grasshopper serum or affinity purified hemagglutinin prior to overlayering hemocytes. While a potential opsonic effect is seen for *Nosema* spores incubated in grasshopper serum, no opsonic activity is demonstrated for *Nosema* incubated in purified hemagglutinin prior to overlayering hemocytes. Neither increased immunofluorescence nor increased phagocytosis was observed when hemocytes, *in vitro*, were incubated with purified agglutinin. Phagocytosis of *Nosema* spores is not inhibited by the addition of agglutinin-specific monoclonal antibody to hemocytes. These results indicate that although grasshopper hemagglutinin is demonstrated in a percentage of grasshopper hemocytes, it does not appear to have an *in vitro* role in the recognition of foreign particles by hemocytes. Potential physiological roles of agglutinins in grasshoppers have not previously been described. Results reported here are consistent with the non-opsonic character and hemocytic location of other described insectan agglutinins.

INTRODUCTION

Insects exhibit a defense system which is remarkably effective at protecting them from pathogenic organisms. Because of their huge numbers and extreme species diversity, the importance of this defense system has been increasingly recognized. An understanding of the insect defense responses may provide workers with clues to the origin of vertebrate immunity, and may uncover novel defense reactions as yet undetected in higher organisms (1). In addition, studies on insect defense mechanisms may lead to new biological control agents to help man in his constant battle with insect pests.

Like the vertebrate immune system, insect defense responses involve both cellular and humoral components. Unlike the vertebrates, insects, along with other invertebrates, neither possess immunoglobulins nor the complement system (2). Thus, invertebrate defense reactions do not show the specificity or memory critical to the vertebrate immune system. Invertebrates have, however, developed a defense response that is rapid, efficient, and exhibits a broad degree of specificity. Invertebrates are able to distinguish self tissue from non-self, and a variety of mechanisms have evolved for dealing with the latter.

In invertebrates, and insects in particular, both hemocytes and serum proteins remove foreign objects from circulation. In general, insects exhibit three main mechanisms for dealing with invading microorganisms. These mechanisms include the cellular responses phagocytosis, encapsulation and nodule formation, and the humoral antimicrobial proteins (1,2,3).

Phagocytosis in insects is similar to that of vertebrate macrophages. Insect contain specialized phagocytic cells, generally termed plasmatocytes, which circulate in the hemolymph. In addition, insects often have sessile phagocytic cells found lining the body wall and internal organs. These cells are responsible for removing small foreign particles from circulation. The particles are engulfed by the cell and subsequently destroyed by lysosomal enzymes (for review of invertebrate phagocytosis see 4,5,6).

Encapsulation and nodule formation also are cellular responses involving the circulating hemocytes. Encapsulation generally occurs with foreign particles larger than 10 um (6). Nodule formation is similar to encapsulation and occurs when microorganisms exceed the number that can be cleared by phagocytosis (1). This process involves the formation of large aggregates of particles and hemocytes, usually 5 to 30 layers, packed tightly against the foreign particle. A capsule is formed against an organ or body wall, trapping the invader, thus removing it from

circulation, (1,2,6,7). The adhesion of the hemocytes to the foreign particle is achieved by a variety of sticky molecules formed during hemocyte degranulation. Encapsulation and nodule formation is usually accompanied by melanization of the aggregates. Melanization is the result of the prophenoloxidase enzyme cascade, believed to be triggered by hemocyte degranulation. This cascade produces toxic quinones and melanin, which may then cause microbial death. The prophenoloxidase cascade has been reviewed elsewhere (8,9).

Antimicrobial proteins are a third, humoral, component of invertebrate defense systems. Antibacterial factors have been found in a wide variety of insects and show activity towards both Gram-negative and Gram-positive bacteria. One of the most widespread antibacterial factors in invertebrate hemolymph is lysozyme, which causes the lysis of Gram-positive bacterial cell walls (1). The cecropins and attacins are non-lysozymal lepidopteran antimicrobial proteins that show broad spectrum antibacterial activity (1,10,11). Cecropins have been shown to cause bacterial lysis and may function in membrane disruption (1,11). Additional cecropin-like proteins have been identified in dipterans (12), and have been proposed to be widespread in insects (1). In addition, antiviral hemolymph factors have been identified in some insects (10).

The cellular responses of phagocytosis and encapsulation involve the interaction of foreign particles with hemocytes. A prerequisite for this interaction is the recognition of foreignness. In the vertebrate immune system immunoglobulins and the complement system are responsible for this initial recognition. These components act as opsonins, coating foreign particles and targeting them for removal by the macrophages. How do insects discriminate between self and non-self tissue? Specifically, do invertebrates have molecules which function in the recognition of foreign material?

Invertebrate hemolymph can cause the agglutination of bacteria and vertebrate erythrocytes *in vitro*. This agglutininating activity is due to the presence of soluble polyvalent lectins, called hemagglutinins. As lectins, agglutinins recognize and bind to specific carbohydrate residues present on the surfaces of cells. Lectins have been demonstrated in the hemolymph of a wide variety of invertebrates, including the arthropods (13,14,15), and they are considered to be ubiquitous among living organisms (16,17). In insects, hemagglutinins have been found in grasshoppers (18,19), crickets (20), flesh flies (21), cockroaches (22,23,24,), beetles (25), locusts (26), and moths (27,28,29).

Due to their carbohydrate binding capacity, insect agglutinins have been proposed to be the initial recognitory

step in pathogen clearance (30,31,32,33). Agglutinins, by binding to carbohydrate residues on foreign particles, may serve as the link between the invader and the hemocytes responsible for encapsulation or phagocytosis.

The following models have been proposed for the roles of agglutinin in insect defense responses (7,21,30,31,33).

1. Agglutinins may be membrane bound components of the circulating hemocytes. Agglutinin would then act directly, by binding the foreign particles via surface carbohydrates, causing the adherence of the foreign particle to the hemocyte.

2. Humoral hemagglutinins, circulating in the hemolymph, may serve as opsonins. The agglutinins would bind to and coat foreign particles and as a result of the agglutinin polyvalent nature, bind the invader to a phagocytic hemocyte via specific hemagglutinin carbohydrate receptors located on the hemocyte surface. Thus the foreign particle and hemocyte would be crosslinked by an agglutinin bridge.

3. Humoral hemagglutinins may help neutralize foreign particles by agglutinating the particles into larger aggregates. This would then allow encapsulation or nodulation to proceed more efficiently.

4. Hemagglutinins may be involved in wound healing and metamorphosis by binding disintegrating pieces of tissue thereby targetting them for removal. This mechanism would be especially relevant to holometabolous insects.

5. Hemagglutinins may not be directly involved in insect defense. Rather they may serve as carrier molecules for glycoprotein, glycolipid, and carbohydrate transport processes.

The proposed roles for agglutinin in invertebrate defense systems are mainly inferred from the capacity of invertebrate hemolymph to agglutinate bacteria and vertebrate erythrocytes *in vitro*. In addition, some similar recognitory lectin functions have been demonstrated in vertebrates (31). However attempts to define hemagglutinin function in invertebrates have been inconclusive, and the majority of these studies have centered on noninsect species. These experiments have generally concentrated on two possible roles for hemagglutinin, that of hemocytic membrane-bound agglutinin, and a humoral hemagglutinin-opsonin.

In an attempt to establish the validity of the agglutinin as a membrane-bound component of hemocytes, researchers have tried to localize hemagglutinin on hemocytes. This has been done successfully for three mollusc species (34,35,36). Using immunochemical techniques, hemocytes from the snail *Lymnaea stagnalis*, (34) and the oyster *Crassostrea virginica* (36) have been demonstrated to contain a cell surface hemagglutinin. In both cases antibodies directed toward the hemagglutinin were prepared by injecting rabbits with serum-agglutinated and washed rabbit erythrocytes. It

was assumed that the hemagglutinin responsible for the erythrocyte clumping was the major antigenic protein that elicited antibody production.

In a similar experiment, Renwrantz and Stahmer (35) demonstrated the presence of a cell membrane hemagglutinin on the hemocytes of the mussel Mytilus edulis. For this experiment, the anti-agglutinin antiserum was prepared by injecting rabbits with Mytilus hemolymph, followed by affinity purification of the antibodies on a Sepharose-agglutinin column.

To date, hemocyte-associated agglutinins have been demonstrated in four insect species (27,37,38,39). Amirante and Mazzalai (37) localized hemagglutinin in the cytoplasm and on the plasma membrane of the granular and spherule hemocytes of the cockroach Leucophaea maderae L., and Yeaton (27) demonstrated an agglutinin on the surface of plasmatocytes and in the cytoplasm of the granular hemocytes of the silkmoth, Hyalophora cecropia. Both of these studies utilized anti-agglutinin antisera prepared in rabbits injected with hemolymph-agglutinated rabbit erythrocytes. Similarly, Lackie (38), using an antibody directed against purified lectin, showed that the hemagglutinin from the cockroach Periplaneta americana is associated with all hemocyte types in that insect.

Using an alternative technique, Komano and associates (39) demonstrated hemocyte-associated hemagglutinin in the

flesh fly Sarcophaga perigrina. Purified flesh fly agglutinin was radioiodinated with ^{125}I. This labelled lectin was then shown to bind to hemocytes in vitro. This association with hemocytes appears to be mediated via lectin-carbohydrate interactions, as binding was completely inhibited by galactose.

The standard method for demonstrating hemagglutinin opsonic activity consists of the addition of serum-treated or serum-untreated test particles to hemocytes in vitro. If opsonic activity is present, serum pretreatment will increase the binding or phagocytosis of the particles. These experiments most often use mammalian erythrocytes as the test particle, thus the role of hemagglutinin as the serum opsonin is inferred from the in vitro agglutination of the test particles. In this manner, a serum opsonin has been demonstrated in the hemolymph of the mussel Mytilus (35) and the snail Lymnaea (40). Similarly, a serum opsonin from the snail Helix pomatia has been studied in vivo. Renwrantz and colleagues (41,42) have demonstrated that the elimination of mammalian erythrocytes and yeast cells from the circulation of the snail is greatly increased by pretreatment of the particles in snail serum. Clearance of these particles can be inhibited by N-acetylated hexosamines. As before, the humoral factors responsible for recognition of the foreign particles were believed to be hemagglutinins.

A more precise determination of the opsonic activity of molluscan hemagglutinin has been attempted by Renwrantz and Stahmer (35). The agglutinating activity in Mytilus hemolymph was isolated by affinity chromatography on Sepharose-mucin. This purified material, which still exhibited erythrocyte agglutination, was then used to pretreat yeast cells prior to phagocytosis assays. Mytilus hemocytes phagocytosed agglutinin-incubated yeast cells much more readily than saline-incubated yeast cells. The phagocytosis stimulating activity of the isolated hemagglutinin was comparable to the activity of Mytilus hemolymph. In Mytilus, hemagglutinin appears to have opsonic properties in vitro.

Attempts to demonstrate the presence of hemolymph opsonizing factors in insects have been unsuccessful (43,44,45). Insects studied for the presence of serum opsonins have been the cockroaches Blaberus craniifer (43), and Periplaneta americana (44,45), and the stick insect Clitumnus extradentatus (45). In each case hemocytes, incubated in vitro, with hemolymph-pretreated particles failed to show increased adherence or phagocytosis. In fact, Scott (44) and Rowley and Ratcliffe (45) found that sheep erythrocytes incubated in hemolymph adhered less avidly to hemocytes.

In a related study, Pendland and Boucias (29) have shown that a purified lectin from the beet armyworm binds to fungal cell wall surfaces. Binding of the agglutinin to the

fungus was detected by incubating spores with fluorescence-labeled agglutinin. Although binding to the spore would be a prerequisite for hemagglutinin opsonic activity, phagocytosis experiments were not done, leaving the question of the function of the hemagglutinin unanswered.

To date, experiments on insect hemagglutinins have not supported an opsonic role for the lectins. However, no purified hemagglutinins have been tested for opsonic activity. It may be that in vitro opsonic activity of agglutinins in insects is masked when using molecularly complex whole hemolymph preparations. Therefore, use of purified insect serum in phagocytosis studies should prove valuable. In addition, due to the large number of insect species and their morphogenic diversity, opsonization studies in only three species cannot give generally conclusive results. Additional studies on insect hemagglutinins are necessary before generalizations on their role in defense reactions can be made.

Grasshoppers (Melanoplus differentialis and Melanoplus sanguinipes) contain a hemolymph hemagglutinin (GHA) which agglutinates humanasialo erythrocytes as well as weakly agglutinating several other animal erythrocytes (18,19). Biochemical characterization has shown that a single hemagglutinin accounts for all observed hemagglutination activity (46). The hemagglutinin has been purified by affinity chromatography and exists as a 600-700 Kd molecular

weight noncovalent aggregate of 70,000 molecular weight subunits (46). Hemagglutination of asialo human erythrocytes can be inhibited by a broad range of carbohydrates including D-galactosidic and D-glucosidic residues. Grasshopper agglutination can also be inhibited by EDTA and shows a requirement for calcium (19,46). Mono-specific rabbit polyclonal antibody and a mouse monoclonal antibody directed towards purified M. differentialis agglutinin have been developed. Both antibodies show specificity towards the 70,000 molecular weight hemagglutinin subunit on Western blots of M. differentialis and M. sanguinipes whole hemolymph (47).

Descriptions of the possible role of grasshopper hemagglutinin in the insect's defense system have not been reported in the literature. I propose to study this role using an approach similar to that used for the molluscan and insectan species described above. The availability of agglutinin-specific antibodies and purified GHA should provide this study with more definitive probes in the examination of possible roles for grasshopper hemagglutinin in insect defense mechanisms.

RESEARCH OBJECTIVES

The specific objectives of this study are:

1. To localize grasshopper hemagglutinin in hemocytes from *Melanoplus differentialis* and *Melanoplus sanguinipes*. This will be achieved by the development and application of appropriate immunochemical labeling procedures to detect the hemagglutinin.

2. To examine the possible opsonic character of insect hemagglutinin using the hemolymphatic hemagglutinin isolated from *M. differentialis*. This will be achieved by studying hemocytic adherence or phagocytic response to particles pretreated with hemolymph or purified hemagglutinin.

MATERIALS AND METHODS

Purification and Detection

Collection of Hemolymph

Adult *M. differentialis* and *M. sanguinipes* were provided from permanent colonies at the USDA Rangeland Insect Laboratory, Bozeman, MT. Hemolymph was collected by a capillary pipette from individual cold-anaesthetized insects as previously described (18). The hemolymph was pipetted into an equal volume of cold Dulbecco's phosphate buffered saline (DPBS) (1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.135 M NaCl, pH 7.2) which contained 0.001 M phenylthiourea (PTU) to inhibit melanin formation and 0.001 M phenylmethylsulfonyl fluoride (PMSF) to inhibit proteolytic destruction. Hemocytes and coagulated material were removed by centrifugation at 3000 x g and the hemolymph supernatant solution was stored at -20°C.

Hemagglutination Assay

Preparation of Asialo Human Erythrocytes. Human O^+ erythrocytes were obtained from Bozeman Deaconess Hospital (Bozeman, MT). Erythrocytes were washed four times by centrifugation at 4°C in DPBS. Asialo human erythrocytes (AHRBC) were prepared by incubating erythrocytes for 1 hr at

37°C with 2 mg neuraminidase (type V, Sigma Chemical Co., St. Louis, MO) in 10 ml DPBS, pH 5.7. The asialo erythrocytes were washed four times by centrifugation at 4°C in DPBS prior to use.

Hemagglutination Assay. Hemagglutination activity was assayed by serial two-fold dilution of 25 ul hemagglutination sample (whole hemolymph or purified agglutinin) with 25 ul DPBS using plastic V-bottom microtiter dishes (Dynatech Labs, Inc., Alexandria, VI). and addition of 25 ul of a 2.5% suspension of asialo human erythrocytes. Agglutination was visually determined after 1 hr. The reciprocal of the highest dilution causing agglutination of erythrocytes was the hemagglutination titer.

Purification of Grasshopper Hemagglutinin

Affinity Chromatography. The purification method outlined below represents a slight modification of a procedure reported elsewhere (46). Grasshopper hemagglutinin was purified by affinity chromatography on a column of Sepharose-galactose. A 0.5 x 2.5 cm column (0.5 ml) of Sepharose-galactose was prepared and washed with 50 volumes of DPBS. Approximately 35 ml M. differentialis hemolymph was passed through the column (6 ml/hr) at 25°C. The column was then washed with HEPES buffer (0.01 M N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 0.15 M NaCl, 0.9 mM

$CaCl_2$, pH 7.2) until the 280 nm absorbancy of the effluent returned to near zero. Bound hemagglutinin was released from the column upon elution with HEPES buffer containing 0.1 M alpha-methyl-D-galactoside. The elution buffer was added in 1.5 ml aliquots, with the column stopped between additions and allowed to incubate for 2 hours at 25°C. Hemagglutinin activity of the aliquots was determined by hemagglutination assay using asialo human O^+ erythrocytes as described above.

In addition to purifying hemagglutinin by this modified technique, hemagglutinin was isolated by the previously reported procedure. In the original procedure, all column washes were in DPBS, and elution of agglutinin was performed in DPBS containing 0.2 M galactose.

Concentration. Purified hemagglutinin samples obtained from the above procedure were concentrated to remove the eluting carbohydrate. The agglutinin was concentrated approximately 20 fold in DPBS using a 30,000 molecular weight cutoff microconcentrator (Amicon, Corp., Danvers, MA). The concentrate was then returned to the original volume with DPBS and titered to determine hemagglutination activity.

Production of Agglutinin-specific Antibodies

Monclonal Antibodies. Agglutinin specific monoclonal antibodies (MAB) were prepared previous to this study (47). Purified *M. differentialis* hemagglutinin was prepared by

affinity chromatography and used to immunize mice. Mouse spleen cells were then harvested and fused with mouse X-63 myeloma cells. Hybrids were selected and cloned according to the methods of Kohler and Milstein (48). Hybridomas producing hemagglutinin specific antibody were identified by detection of hemagglutinin on Western blots. Hybridoma supernates from these clones were collected and concentrated 5-fold in an ultrafiltration cell (Amicon Corp., Danvers, MA). Concentrated supernatant solutions were stored at -50°C. Frozen supernates were thawed and dilutedin an appropriate buffer to return them to their original concentration prior to use.

Rabbit Polyclonal Antibodies. During the course of this study, monospecific rabbit polyclonal antibodies were prepared in our lab by a coworker (47). Two female New Zealand white rabbits were immunized with 100 ug purified M. differentialis hemagglutinin according to the multiple intradermal injection method of Vaitukaitis (49). The affinity purified antigen (agglutinin) was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and electro-transfered to nitrocellulose paper. The agglutinin-bearing nitrocellulose was dissolved in dimethyl sulfoxide (DMSO) according to the method of Knudsen (50). The solubilized antigen was then emulsified in an equal volume of complete Freund's adjuvant and injected into the animals at weekly intervals. For each rabbit, control

serum was obtained before injection, and antiserum was harvested 8 weeks after immunization. Specificity of the antiserum was confirmed by Western blots of hemolymph and purifed agglutinin.

Detection of Grasshopper Hemagglutinin

Western Blot. Grasshopper hemolymph and purified hemagglutinin were subjected to electrophoresis in SDS-polyacrylamide gel slabs using the apparatus and procedures from Hoefer Scientific (San Francisco, CA). The stacking gel was 4%, pH 6.8, and the separating gel was 7.5%, pH 8.8, according to Laemmli (51). Electrophoresis was at 15 mA/gel until the proteins entered the separating gel (approximately 1 hr), then amperage was increased to 30 mA/gel for 3 hr.

Proteins separated by SDS-electrophoresis were transferred from the polyacrylamide gel onto nitrocellulose filter paper (Schleicher and Schuell, Keene, NH) using a Hoefer TE 42-Transphor Electrophoresis Cell. For review, see Gershoni and Palade (52). Proteins were blotted in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 90 minutes at a current of 0.8 A. Following transfer, the nitrocellulose was stained either with Amido Black (0.1% Amido Black 10B in 25% 2-propanol, 10% acetic acid, destained with 25% 2-propanol, 10% acetic acid), or by immunochemical means with the agglutinin-specific MAB, followed by a horseradish peroxidase (HRP) labelled secondary antibody (Appendix A).

Con A Probing. Purified grasshopper hemagglutinin subjected to electrophoresis and blotted to nitrocellulose was probed with biotinylated-Concanavalin A (EY Labs, San Mateo, CA) to detect possible contaminating glycoproteins. The nitrocellulose was incubated in a 5 ug/ml dilution of Con A in Tris-tween buffer (0.05 M Tris, 0.5 M NaCl, 0.05% Tween-20, pH 7.2) using the Hoeffer PR-200 decaprobe. The nitrocellulose was then given three 5 minute washes in Tris-tween, followed by incubation in HRP-conjugated Avidin (EY Labs) at a concentration of 1 ug/ml in Tris-tween. Simultaneous with this incubation, additional nitrocellulose lanes were incubated in the agglutinin-specific immunochemicals, and all lanes were then developed as described (Appendix A).

Silver Stain. Hemagglutinin purified by the modified affinity chromatography procedure was subjected to SDS-polyacrylamide electrophoresis, with subsequent silver staining of the gel. The silver stain method of Morrissey (53) was employed.

Localization

Hemolymph Probing

To determine possible *in vivo* receptors for GHA, hemolymph from *M. sanguinipes* was subjected to SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose. Individual sample lanes in the Hoefer decaprobe were then

incubated for 2 hr in either M. sanguinipes whole hemolymph (diluted 1:1 in Tris-tween), or left untreated. The nitrocellulose was given three 10 minute washes in Tris-tween, then all lanes were immunostained with horseradish peroxidase labeled conjugate as described in Appendix A.

Formation of Hemocyte Monolayers

Hemocyte monolayers were prepared in either 24 well plates (Nunclon, Denmark), or on 20 mm square glass coverslips placed inside 35 mm tissue culture dishes (Becton Dickinson Labware, Lincoln Park, NJ). Adult grasshoppers were bled by capillary pipette as previously described. The hemolymph was pipetted into the culture dishes containing 1 ml cold DPBS to which had been added 4 ul/ml glycerol (to increase osmolarity to 400 mOsm), and 0.65 mg/ml glutathione (to prevent melanization). After 20 minutes at room temperature the hemocytes had spread and adhered to the bottom of the wells or glass coverslips. The hemocyte monolayer was then washed, by Pasteur pipette, with DPBS (adjusted for osmolarity) to remove hemolymph and non-attached cells.

Lysing Hemocyte Monolayers

Hemocyte monolayers formed in 24 well plates were lysed for subsequent Western blot analysis. Lysis was carried out by incubating monolayers for 1 hr in 100 ul 2% Nonidet P40 (NP-40; Bethesda Research Laboratories, Gaithersburg, MD) in

DPBS. Lysates were stored at -20°C until subjected to electrophoresis.

Indirect Immunofluorescent Staining (IFA)

Fixed Monolayer Hemocytes. Generally, the method of Willingham and Pastan (54) was followed, and the staining procedure is outlined in Appendix B. Monolayer hemocytes were fixed in 3.7% formalin in DPBS for 1 hr, 4°C. Monolayers were then incubated in the concentrated monoclonal antibody supernates, diluted to their original volume with DPBS. The monolayers were then washed, then incubated in a fluorescein conjugated-goat-anti mouse IgG (Hyclone Labs, Logan UT). The cells were again washed and mounted on glass slides for observation.

Fixed Hemocyte Suspensions. Hemocyte suspensions were prepared by bleeding cold-anaesthetized grasshoppers directly into polypropylene tubes containing 1 ml ice cold 3.7% formalin in DPBS. Fixation continued for 1 hr at 4°C. The hemocytes were then washed three times by gentle centrifugation (950 x g) and resuspended in DPBS. Suspended hemocytes were stained under identical conditions as that outlined for monolayers, with all incubations occurring in polypropylene tubes on a rocking table.

Live Monolayer Hemocytes. Live hemocytes were subjected to the indirect immunofluorescence protocol under similar conditions as for fixed monolayer hemocytes, with

the following exceptions. All solutions were adjusted to 400 mOsm by the addition of glycerol, and all incubations occurred at 4°C. In addition, the monoclonal antibody solution was heat treated (56°C for 30 minutes) to inactivate complement.

Horseradish Peroxidase Enzyme Immunoassay (EIA) Staining

Indirect Enzyme Immunoassay. Staining procedure for the indirect EIA technique was similar to that for immunofluorescence (Appendix B). Fixed monolayer hemocytes were incubated in monoclonal antibody, washed, then incubated in a horseradish peroxidase-conjugated secondary antibody. The HRP-conjugated goat anti-mouse IgG (BioRad, Richmond, CA) was diluted 1:100 in complete Iscove's Modified Dulbecco's Medium (IMDM) (Gibco Laboratories, Grand Island, New York). The monolayers were then washed, and incubated in developer solution (0.5 mg/ml diaminobenzidine, DAB, 0.01% hydrogen peroxide, in 0.05 M Tris, pH 7.2) for 30 minutes. The hemocytes were washed for 10 minutes in0.05 M Tris, and mounted under glycerol diluted 1:1 with DPBS.

Avidin-Biotin Complex. Monolayer hemocytes were stained for agglutinin using the reagents and protocol of an avidin-biotin complex (ABC) kit (Vectastain Laboratories, Burlingame, CA). Fixed hemocytes were first preincubated in whole goat serum, followed by two five minute washes in DPBS. The monolayers were then incubated in monoclonal

antibody for 1 hr, followed by three five minute DPBS washes. Biotinylated-goat anti-mouse IgG (1:100 in complete IMDM, 0.1 M galactose) was used as the secondary conjugate. The monolayers were washed in DPBS for 15 minutes and incubated in the Vectastain biotin/avidin complex for 30 minutes, according to kit directions. The cells were again washed for 15 minutes and developed in DAB substrate for 30 minutes. After an additional 10 minute wash in DPBS the cells were mounted under glycerol-DPBS.

Immunocytochemical Controls

Controls for nonspecific antibody binding were included for each immunocytochemical labeling experiment. In place of the primary antibody, control antibodies were either normal mouse serum (Cappel Laboratories, Cochranville, PA) at 1 ug/ml in complete IMDM, or an irrelevent mouse monoclonal antibody supernate prepared by Dr. Mickey McGuire (USDA Rangeland Insect Laboratory, Bozeman, MT).

Photomicroscopy

All hemocytes were observed under either phase contrast or 490 nm blue light (for FITC fluorescence) using an Olympus BH-2 microscope with a reflected light fluorescence attachment. Black and white photographs were taken with Kodak 400 ASA Tri-X pan film. Color photographs were taken with Kodak 400 ASA Ektachrome. Exposure times for fluorescence varied from 30 to 60 seconds, depending upon

the intensity of the stain.

Opsonic Characterization

Phagocytosis by Monolayer Hemocytes

Test Particles. Live monolayer hemocytes were incubated with the following particles: Asialo human erythrocytes (HRBC) at a concentration of 1000 HRBC: 1 hemocyte; *Nosema locustae* spores, 20 spores: 1 hemocyte; and *Bacillus thuringiensis* bacteria, 100 bacteria: 1 hemocyte. All incubations were performed in moist chambers at room temperature for 90 minutes. The hemocytes were then washed to remove nonadhering particles, and were observed for particle adherence or phagocytosis by microscopic examination.

Immunostain of Phagocytic Hemocytes. Hemocytes incubated with *Nosema* spores were also subjected to indirect immunofluorescence staining. The hemocytes were incubated in *Nosema* for 90 minutes, washed, and fixed in 3.7% formalin in DPBS. The hemocytes were then probed for the presence of agglutinin as described (Appendix B).

Carbohydrate Inhibition. Dependence of particle adherence or phagocytosis on carbohydrates was determined by incubating the particles on the monolayer in the presence of various agglutinin-specific carbohydrates (as determined by Stebbins and Hapner, 46). Hemocytes were incubated in the

carbohydrates (0.1 M in DPBS) for 15 minutes, then the particles were added to the monolayers in the presence of carbohydrate. Controls consisted of monolayers in which a non-inhibitory carbohydrate was used for incubation, and monolayers in which DPBS was substituted for the carbohydrate solution.

Opsonization. Opsonic properties of hemagglutinin were studied by overlayering live monolayer hemocytes with erythrocytes, Nosema spores, or B. thuringiensis bacteria that had or had not been previously incubated in varying concentrations (1:8, 1:32, and 1:256 in DPBS) of whole hemolymph or affinity purified agglutinin. Pretreated particles were incubated in whole hemolymph or purified agglutinin for 30 minutes, then overlaid on the monolayer while still in the incubating solution. In a second experiment, particles were pretreated as before, only subjected to a wash by centrifugation and resupended in DPBS prior to overlayering the monolayer. Due to the tendency of erythrocytes to clump during some of the hemolymph and agglutinin incubations, erythrocytes were ejected through a 23 gauge needle prior to overlaying the monolayers. Control particles were incubated in DPBS, otherwise treated identically to the experimental particles.

Hemocytic Agglutinin Receptors. Monolayers of live hemocytes were incubated for 30 minutes in purified

hemagglutinin (approximately 5 ug/ml in DPBS) prior to overlayering with erythrocytes and Nosema spores.

Live hemocytes incubated with agglutinin were also subjected to immunofluorescence assay to detect possible hemocytic agglutinin binding sites. After incubation in agglutinin the hemocytes were fixed in 3.7% formalin-DPBS and subjected to the normal immunofluorescence protocol (Appendix B).

Blocking of Phagocytosis with Immunochemicals. Live monolayer hemocytes were preincubated in monoclonal antibody and Nosema spores were then added to the monolayer while still in antibody solution. The monolayers were incubated in a moist chamber for 1 hr. As a negative control, additional monolayers were incubated in mouse whole serum (1 ug/ml in complete IMDM) instead of MAB.

Data Analysis

Six to ten female M. differentialis adults were used to form the monolayers for each of the above opsonization experiments. The percentage of hemocytes with adhering or phagocytosed particles and the average number of particles per hemocyte with one or more particle was determined for each monolayer by observing 500 hemocytes in randomly selected microscopic fields. Levels of significance for difference of means were determined using the Student-t test. The level of significance for each experiment was $p<0.05$.

RESULTS

Purification and Detection

Purification of Grasshopper Hemagglutinin

Grasshopper hemagglutinin is routinely purified in our laboratory by affinity chromatography on Sepharose-galactose. Previous purifications have used 0.2 M D-galactose in DPBS as the elution buffer, and on average isolates approximately 350 ug protein. Typical results have been published elsewhere (46). For the purposes of this study the technique was modified by incorporating 0.1 M alpha-methyl-D-galactoside in 0.1 M HEPES as the elution buffer. Alpha-methyl-D-galactoside was added to the column in aliquots, and incubated for several hours prior to elution, as opposed to a continuous elution with galactose. Hemolymph prior to application to the column had a hemagglutination titer of 4096. The titer value of hemolymph emerging from the column was 32, indicating that approximately 99% of the original activity was bound to the affinity matrix. Typical hemagglutination activity of the purified agglutinin aliquots was in the range of 4096-8192. This technique resulted in the isolation of about 700 ug of hemagglutinin from 35 ml *M. differentialis* hemolymph sample, as determined by a modified Lowry protein assay

(55). This is double the yield of the previous purification method, and is due to the incorporation of alpha-methyl-D-galactoside, a more effective competitor for the lectin, in the elution buffer, and the increased time of elution used.

After concentrating and washing the purified agglutinin to remove the carbohydrates used for elution, the titer dropped to 512-1024, representing an 8-fold loss in hemagglutination activity. This loss in activity is presumably due to agglutinin precipitating on the microconcentrator membrane.

Immunochemical Detection of Grasshopper Hemagglutinin by Western Blot Analysis

Both the agglutinin-specific monoclonal antibody, and the rabbit polyclonal antibody detect hemagglutinin in Western blots of whole hemolymph and purified agglutinin preparations. Staining of hemagglutinin is specific, as no other hemolymph proteins are immunostained. Western blotting and immunochemical staining of hemagglutinin can provide an accurate and sensitive technique for the detection of agglutinin in complex mixtures of proteins.

M. differentialis hemagglutinin is often detected as a doublet band of approximate molecular weights 59,000 and 53,000 (Fig. 1). *M. sanguinipes* hemolymph contains an antigenically similar hemagglutinin that also immunostains as a double band of molecular weights 59,000 and 53,000. These two bands indicate a slight agglutinin heterogeneity

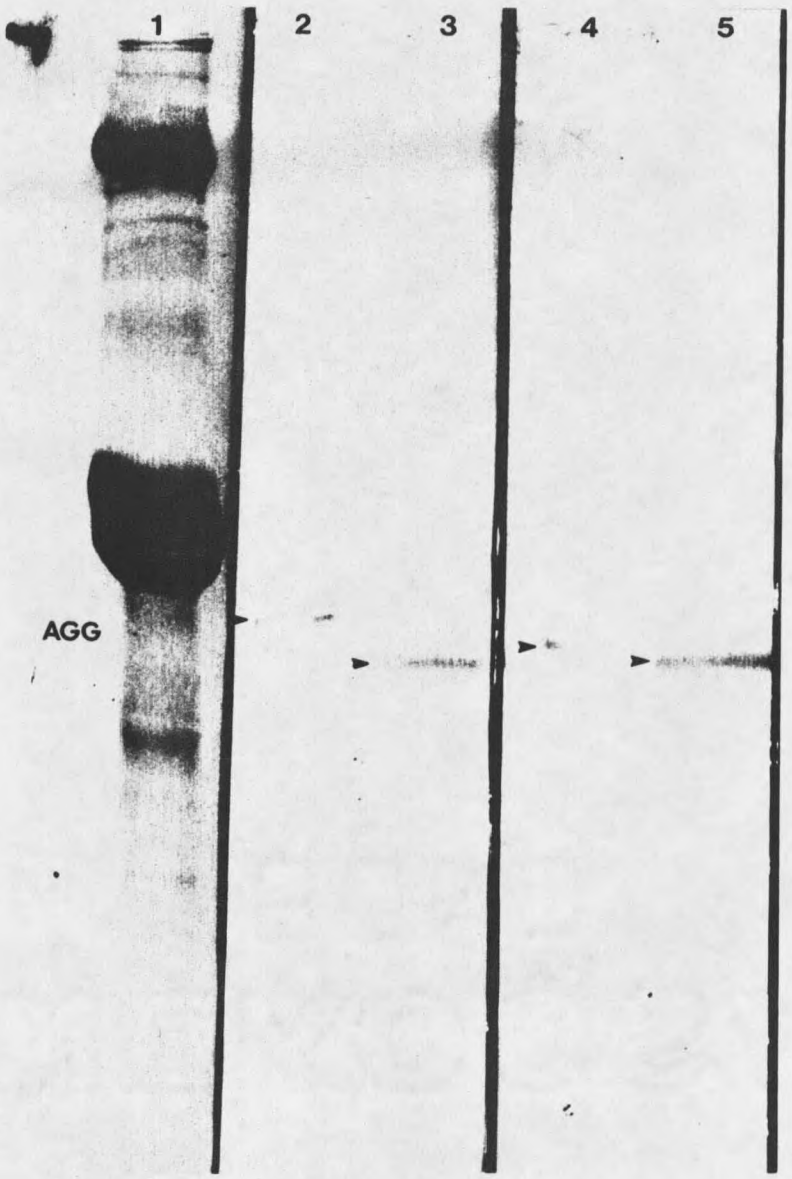

Figure 1. Western blot of whole grasshopper hemolymph and affinity purified agglutinin showing agglutinin-specific staining by both mouse monoclonal and rabbit polyclonal antibodies. Lanes 1,2,4 contain hemolymph, lanes 3,5 purified hemagglutinin. Lane 1 was stained with Amido Black, lanes 2,3 with monoclonal antibody, lanes 4,5 with rabbit polyclonal antibody (AGG-agglutinin).

in grasshoppers. These results are slightly different from previously published determinations of the grasshopper hemagglutinin molecular structure. Earlier, Stebbins and Hapner (46) had reported a single band of molecular weight 70,000 when purified hemagglutinin was examined by electrophoresis in SDS polyacrylamide gels and stained with Coomassie Blue G-250. In their procedure, electrophoresis was carried out at 30 mA/gel for 3 hr, while in the presently reported procedure electrophoresis was at 15 mA/gel for 1 hr, then 30 mA/gel for 3 hr. This initial low amperage allows for better stacking of the proteins at the stacking-separating gel interface. This would then allow for greater protein separation and may cause the increased disassociation of hemagglutinin from the other hemolymph components. Thus the molecular weight of SDS-denatured hemagglutinin may be more accurately represented as 53,000 to 59,000.

Concanavalin A probing

Seven horseradish peroxidase stained bands were observed when hemagglutinin purified by the techniques previously reported in the literature (46) was subjected to a modified Western blot-Con A probe (Fig. 2). One of these bands may correspond to the immunochemically detected agglutinin band. These bands represent humoral molecules bound by Con A. Con A is a plant lectin specific for alpha-D-mannose and glucose residues. The Con A bands presumably

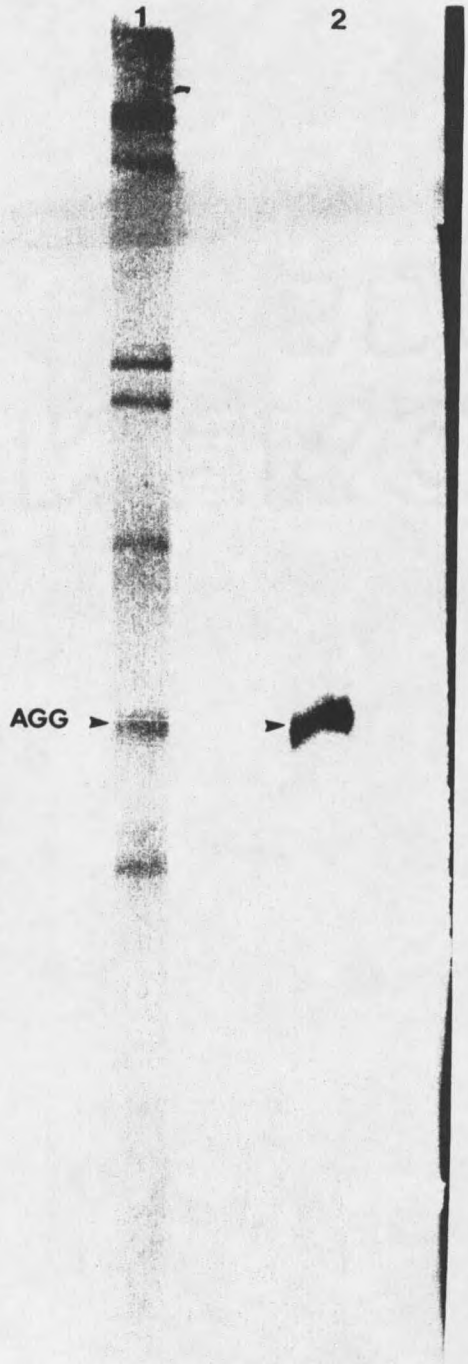

Figure 2. Modified Western blot-Con A probe of purified grasshopper hemagglutinin. Lane 1 contains purified agglutinin incubated in Concanavalin A, lane 2 contains purified hemagglutinin immunostained with monoclonal antibody (AGG-agglutinin).

represent glycoproteins that were copurified with hemagglutinin. A similar probe of hemagglutinin isolated by the modified technique reported in this study identified no Con A binding sites, indicating no detectable glycoprotein contaminants. Thus, the modified purification technique may represent a significant improvement over the previously reported purification method.

Silver Stain

A silver stain of SDS polyacrylamide gels containing the *M. differentialis* hemagglutinin purified by the modified technique reported in this study produced one major protein band of molecular weight 59,000, and a minor band at 56,000 M.W. (Fig. 3), both of which have been shown to be antigenic on Western blots of GHA (47). At loads of 5 ug protein per lane, only one slight contaminant was detected, at a molecular weight of 70,000. The modified purification technique thus provides a convenient method to obtain pure grasshopper hemagglutinin for subsequent opsonization studies.

Localization

Hemolymph Probing

Potential *in vivo* binding sites for grasshopper hemagglutinin were assayed by a modified Western blot-hemolymph probing experiment (Fig. 4). Electrophoresed *M. sanguinipes* hemolymph was blotted to nitrocellulose, then incubated in additional *M. sanguinipes* hemolymph. Upon

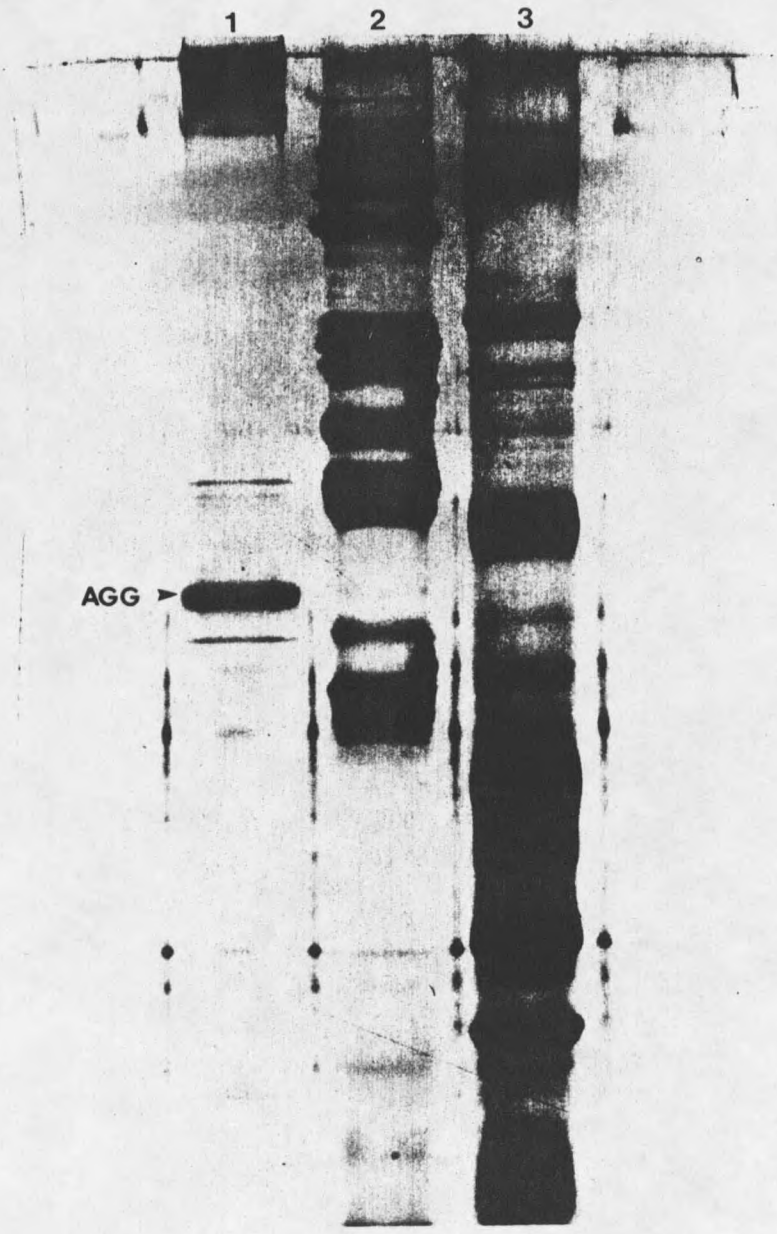

Figure 3. SDS-polyacrylamide gel electrophoresis followed by silver stain of whole grasshopper hemolymph and affinity purified hemagglutinin. Lane 1 contains 30 ug of affinity purified hemagglutinin. Lane 2 contains grasshopper hemolymph and lane 3 contains molecular weight markers (AGG-agglutinin).

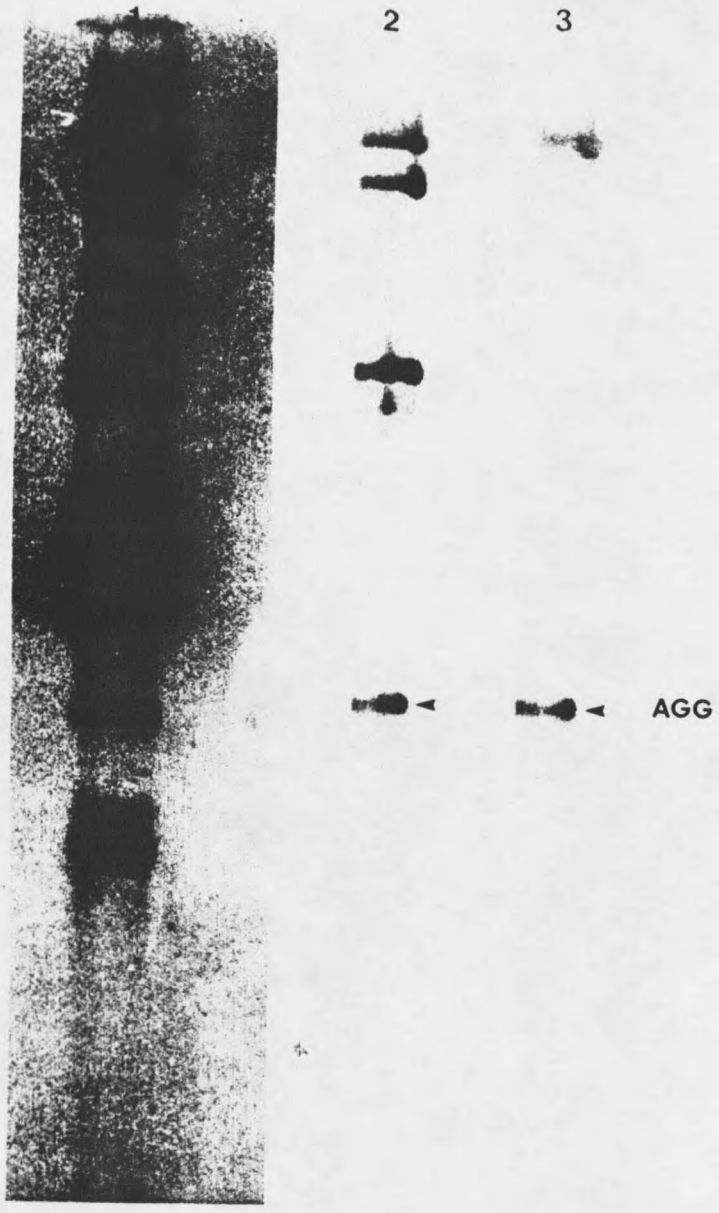

Figure 4. Modified Western blot and hemolymph probing experiment. Lane 1 contains whole hemolymph stained with Amido Black, lane 2 contains whole hemolymph incubated in whole hemolymph prior to immunostaining, and lane 3 contains whole hemolymph that was immunostained (AGG-agglutinin)

immunostaining, six bands were identified. One of these bands corresponded to the hemagglutinin band typically seen in Western blots of whole hemolymph. The additional bands were sites where hemagglutinin has bound to other hemolymph components during the hemolymph probing step. These bands may represent possible humoral receptors of hemagglutinin.

Immunochemical Localization of Agglutinin in Hemocytes

These results describe localization of hemagglutinin in *M. differentialis* hemocytes. Similar studies with *M. sanguinipes* hemocytes were performed and yielded virtually identical results.

Description of Monolayer Hemocytes

Grasshopper hemocytes strongly adhere to both plastic culture dish surfaces and glass coverslips. Monolayers formed on glass show slightly better definition of the hemocyte membrane and membrane extensions. Monolayers formed on glass coverslips were then used for the following studies. Generally, two types of hemocytes are seen in grasshopper monolayers (Fig. 5). Granular cells (granulocytes) are the most prominent and often constitute approximately 95% of the monolayer cells. These cells are characteristically granular and often spread in a fibroblast-like appearance. Granular cells average 10-30 um in diameter. Granular cells are relatively unstable, and live granulocytes will easily burst in the presence of whole

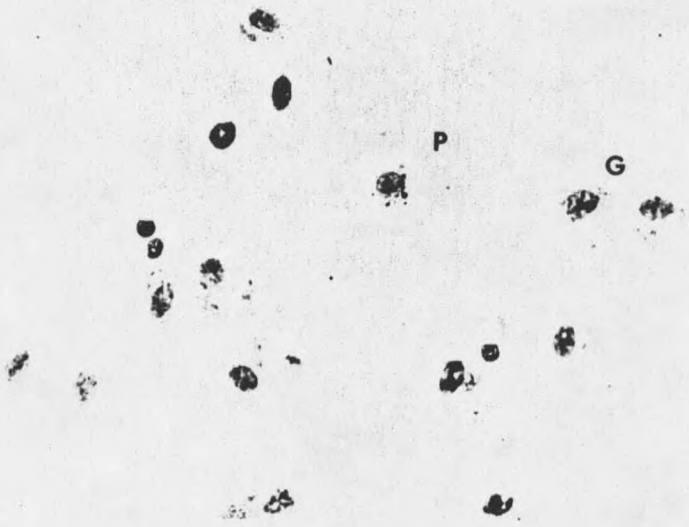

Figure 5. Phase contrast photomicrograph of monolayer hemocytes from M. differentialis. G-granulocyte, P-plasmatocyte (250x).

hemolymph or antibody solutions. In contrast, plasmatocytes are few in number, often numbering only 4-8% of the cells in the monolayer. Plasmatocytes are agranular, and are more stable than granular cells. Plasmatocytes can be as large as 40-50 um in diameter, have a centrally located nucleus, and often display a ruffled outer membrane. This can give these cells a fried-egg appearance. For a comprehensive review of insect hemocytes see Gupta (56).

Western Blots of Hemocyte Lysates.

Monolayer hemocytes were lysed in 2% NP-40, which disrupted and solubilized the cell membrane and cytoplasm, but left the nucleus intact. This provided a nonviscous lysate which was amenable to SDS-PAGE. Figure 6 shows the results of a Western blot analysis of individual female M. differentialis hemocyte lysates. A doublet band of molecular weights 59,000 and 53,000 was detected by the agglutinin-specific monoclonal antibody. This indicates that grasshopper hemocytes do contain hemagglutinin which is similar in electrophoretic pattern to the humoral hemagglutinin.

Immunofluorescent Localization

Unsuitablility of Rabbit Antisera. Mouse monoclonal antibody was the antibody used in all the following immunocytochemical localization experiments due to high background fluorescence encountered with the rabbit

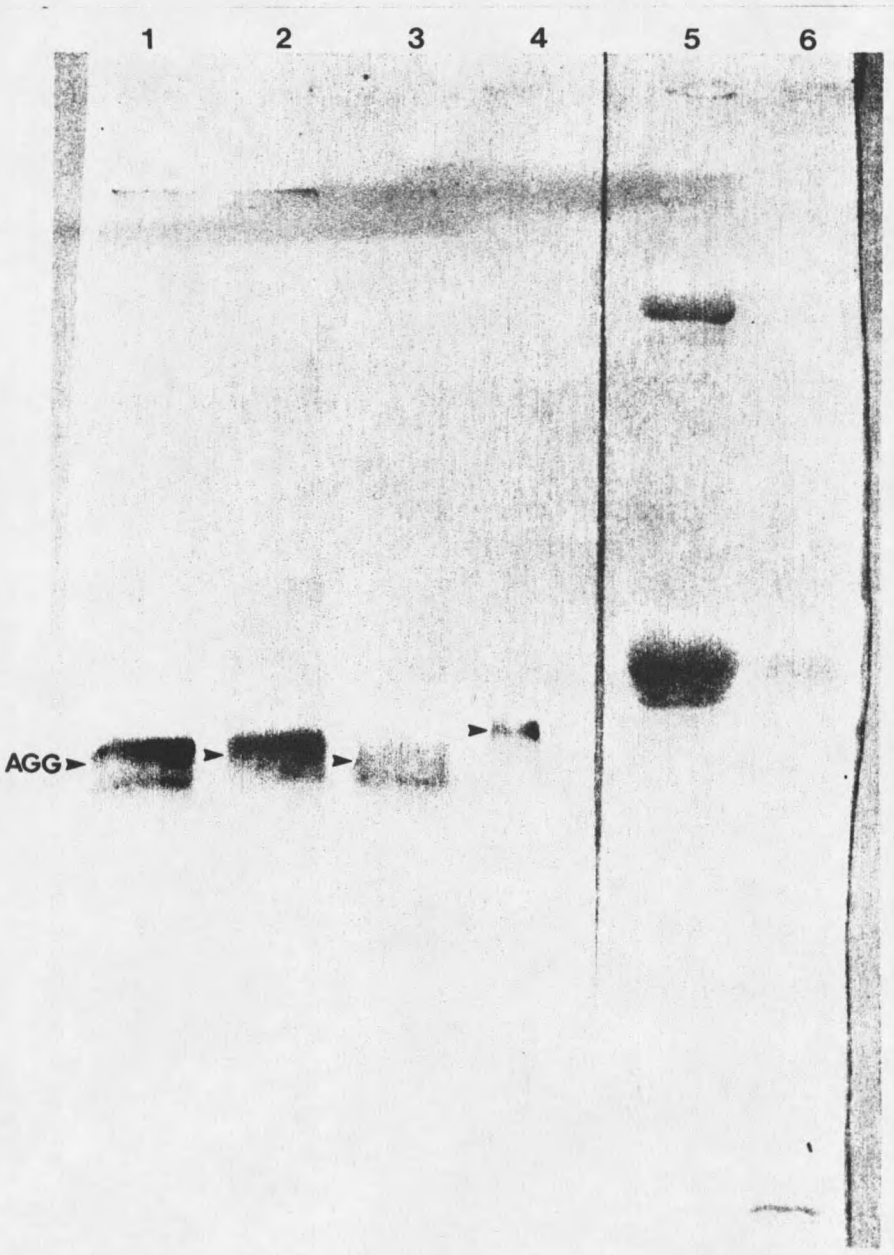

Figure 6. Western blot of hemocyte lysates. Lanes 1,2,3,6 contain M. differentialis hemocytes lysed with 2% NP-40. Lanes 4,5 contain whole grasshopper hemolymph. Lanes 5,6 were stained with Amido Black, lanes 1,2,3,4 were probed with mouse monoclonal antibody followed by HRP-conjugated secondary antibody (AGG-agglutinin).

antisera. Fixed monolayer hemocytes subjected to the immunofluorescence protocol using the rabbit antisera as the primary antibody showed no difference in staining intensity over control hemocytes incubated in rabbit preimmune serum. Purified rabbit IgG at 1 ug/ml was also observed to bind nonspecifically to grasshopper hemocytes, producing high background fluorescence. This non-specific binding of rabbit IgG to hemocytes could not be inhibited by changing the fixative to methanol, or by the addition of 0.2 M galactose or 10 mM EDTA to the antibody solutions. Similar non-specific binding was observed for live hemocytes incubated in rabbit immunoglobulins. Non-specific binding of purified goat or mouse IgG was never shown to be a problem. It appears that grasshopper hemocytes contain a receptor specific for rabbit gamma globulin, possible similar to vertebrate Fc receptors. This makes rabbit immunoglobulins unsuitable for immunocytochemical staining techniques in grasshoppers.

Monolayer Hemocytes. Figure 7 shows a series of photomicrographs of fixed monolayer hemocytes that were subjected to the indirect immunofluorescence staining process. Hemocytes that contain the hemagglutinin exhibit bright apple-green fluorescence, characteristic of fluorescein. Fluorescence appears to be cytoplasmic, with areas of intense, granular, staining. Fluorescent labeling of the hemocyte membrane was not observed. Staining was found only

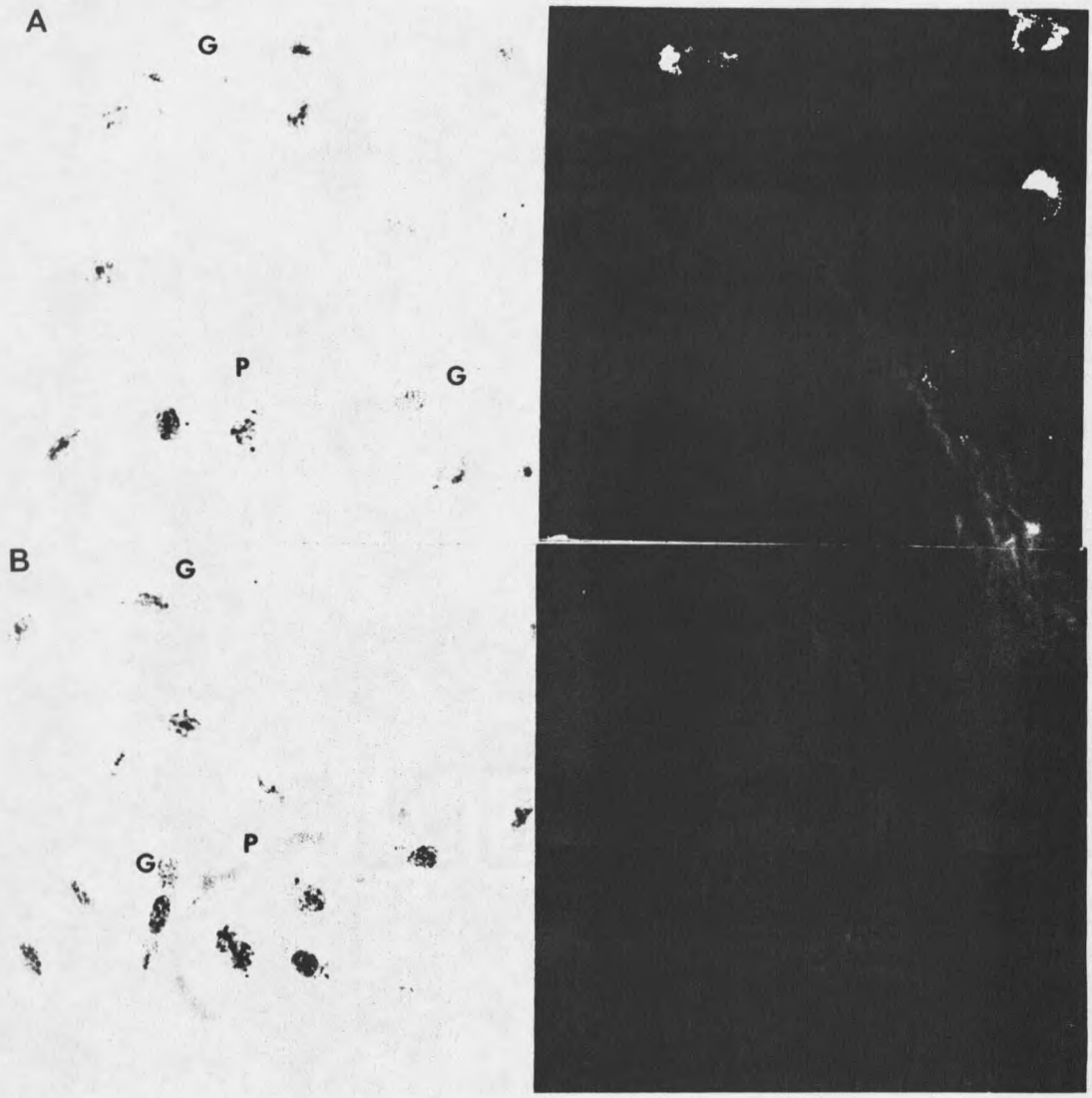

Figure 7. Phase contrast and fluorescence (45 sec, 400 ASA) photomicrographs of fixed monolayer hemocytes from M. differentialis. subjected to indirect immunofluorescence stain for grasshopper hemagglutinin. A-Hemocytes were incubated in agglutinin-specific monoclonal antibody followed by FITC-conjugated secondary antibody. B-Hemocytes incubated in mouse whole serum followed by FITC-conjugated secondary antibody as a negative control (G-granulocyte, P-plasmatocyte; 250x).

among granulocytes, where approximately 22% of the cells stain. This percentage is somewhat variable among individual grasshoppers. Plasmatocytes showed no fluorescent staining. Similar staining patterns were seen among both female and male adults and fifth instar nymphs. Old grasshoppers, and those with typical colony diseases (e.g. Malameba locustae infection) generally show similar staining patterns, although the hemocytes often appear vacuolated.

Suspended Hemocytes. Fixed, suspended hemocytes exhibited immunofluorescent staining results similar to those for monolayer hemocytes. Approximately 20-25% of the hemocytes stain for the presence of agglutinin, and staining is again confined among the granular cell types. Identification of the stained cells was inconclusive, however, as suspended cells were not as easily identifiable as spread monolayer hemocytes.

Live Hemocytes. In an attempt to label agglutinin bound to the hemocyte plasma membrane, live hemocytes were incubated in the agglutinin-specific MAB, followed by the fluorescein-conjugated secondary antibody. Live granular cells are not tolerant of the antibody solutions, and these cells were often observed to burst during the labeling procedure. The remaining intact cells included some granulocytes and the plasmatocytes. These cells did not exhibit

any difference in staining patterns as compared to mouse serum-incubated control hemocytes. As agglutinin was not detected on live hemocytes, this may indicate that agglutinin is absent from hemocyte membranes. However, due to the large percentage of lysed hemocytes, this remains inconclusive.

Controls. Controls used for the immunofluorescence hemagglutinin staining procedures consisted of hemocytes incubated in either mouse whole serum or an irrelevent monoclonal antibody supernate substituted for the agglutinin-specific MAB. This was then followed by the fluorescein-conjugated secondary antibody as for experimental monolayers. Control hemocytes exhibited only very dim fluorescence, indicating a small amount of non-specific staining. In no case were the fluorescent intensity of the controls comparable to that of experimental monolayers.

Horseradish Peroxidase Enzyme Immunoassay Localization

Indirect HRP-EIA. Indirect EIA was used as an additional immunocytochemical labeling approach to confirm the immunofluorescence results describe above. Figure 8 shows the results of an indirect EIA localization of hemagglutinin on monolayer hemocytes. Cells containing hemagglutinin have turned brown, resulting from the reaction of horseradish peroxidase with the substrate DAB. Only the granular cells appear brown under the light microscope,

Figure 8. Phase contrast photomicrographs of fixed monolayer hemocytes from M. differentialis subjected to indirect EIA stain for grasshopper hemagglutinin. A-Hemocytes incubated in agglutinin-specific MAB, B-hemocytes incubated in mouse whole serum followed by HRP-conjugated secondary antibody and developed in hydrogen peroxide-DAB (250x).

plasmatocytes do not appear to stain. Approximately 31% of the granulocytes stain for the presence of agglutinin using this immunocytochemical technique. This is a slightly greater percentage of stained hemocytes as compared to the indirect immunofluorescence staining procedure, indicating that agglutinin localization by EIA may be slightly more sensitive than indirect immunofluorescence (Fig. 9). Control monolayers incubated in mouse whole serum as the primary antibody do not stain, indicating very low, or nonexistent, non-specific staining. The HRP-EIA procedure therefore provides results closely consistent with the immunofluorescence method.

Avidin-Biotin Complex. Fixed monolayer hemocytes stained with the Vectastain ABC kit showed results similar to the HRP-EIA procedure. Only the granular cells showed the brown DAB precipitate. Plasmatocytes did not stain. This is consistent with the HRP-EIA and immunofluorescent staining results. However, the ABC technique did not seem to be as sensitive as the HRP-EIA or immunofluorescent methods, as a much lower percentage of hemocytes were stained. Control monolayers, incubated in mouse whole serum in complete IMDM as a substitute for MAB, exhibited high background staining, indicating non-specific labeling. This may be due to endogenous hemocytic biotin that could bind the avidin-biotin complex. Due to these problems, this technique was discontinued.

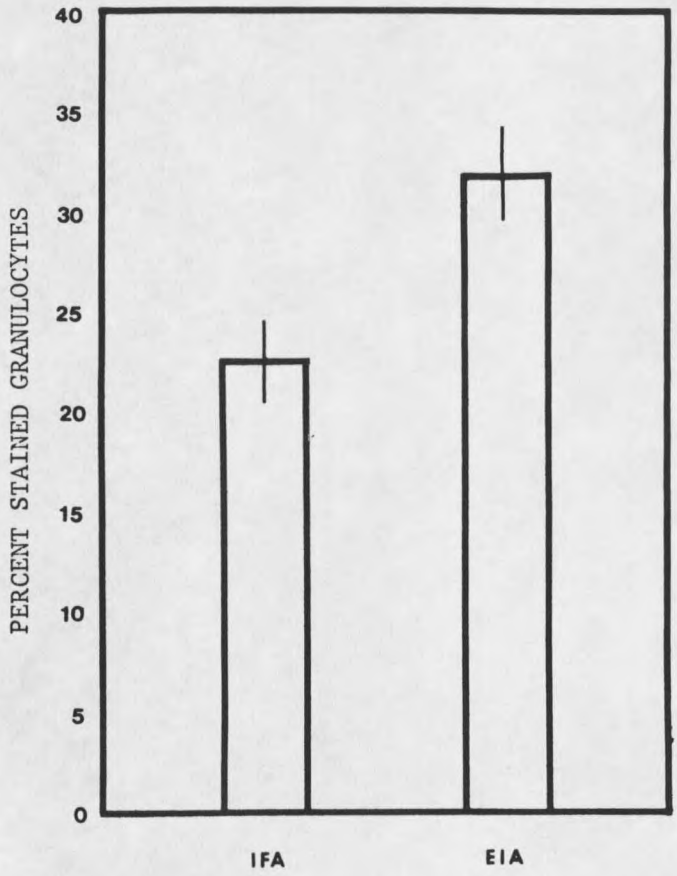

Figure 9. Comparison of indirect immunofluorescence (IFA) and enzyme immunoassay (EIA) methods for detecting grasshopper hemagglutinin in monolayer hemocytes. Mean values $\pm$ 1 S.E.

Opsonic Characterization

Particle Interactions with Hemocytes

Erythrocyte Rosetting. Hemocytes incubated in asialo human erythrocytes bound the erythrocytes at the plasma membrane. The percentage of hemocytes that bound AHRBC was variable, and was observed to be from 10-30% of the hemocytes in the monolayer. Both plasmatocytes and granulocytes were observed to have adhering AHRBC. If erythrocyte concentration was sufficient (1000 erythrocytes: 1 hemocyte) the erythrocytes often surrounded the hemocyte membrane, forming a rosette. Grasshopper hemocytes did not appear to phagocytize the erythrocytes. No internalized AHRBC were observed, as judged by the refractivity of the erythrocytes. In addition, fixing the hemocytes in 3.7% formalin resulted in the loss of all adhering erythrocytes. This indicates that the hemocytes did not phagocytize AHRBC.

Nosema Phagocytosis. Grasshopper hemocytes will avidily phagocytize Nosema spores. Approximately 5-10% of the hemocytes engulf spores, and these are exclusively the plasmatocytes. At concentrations of 20 Nosema per hemocyte, phagocytic hemocytes (plasmatocytes) were observed to engulf 0-20 Nosema spores each. Under these conditions, granular cells did not bind or phagocytize Nosema spores in vitro.

Bacillus thuringiensis Adherence. Approximately 65-75% of live monolayer hemocytes incubated in B. thuringiensis

were observed to bind the bacteria. Bacteria adhered to both granulocytes and plasmatocytes, as did AHRBC. While granulocytes did not appear to phagocytize bacteria, phagocytosis of the bacteria by plasmatocytes was occasionally observed. At concentrations of 100 bacteria per hemocyte, hemocytes that bound bacteria bound on average 3 bacteria each.

IFA Staining of Phagocytic Hemocytes

Live monolayers incubated in Nosema spores were fixed and subjected to the indirect immunofluorescence staining procedure. Hemocytes stained as normal, with the granular cells exhibiting intense cytoplasmic staining. Plasmatocytes did not stain for agglutinin, including those plasmatocytes that had engulfed Nosema spores. The Nosema spores tended to pick up a small amount of non-specific fluorescence in both the monoclonal incubated monolayers and the mouse IgG incubated controls, however in all cases this was discernable from true agglutinin label.

The results of this experiment indicate that phagocytic hemocytes do not contain agglutinin. As plasmatocytes also are capable of binding AHRBC and B. thuringiensis bacteria, cellular hemagglutinin may not be required for particle adherence of phagocytosis by plasmatocytes. However, this conclusion must be tempered by the fact that hemocytes may contain membrane-bound agglutinin that was not detected.

Carbohydrate Inhibition

Hemocyte monolayers overlaid with an erythrocyte solution containing 0.1 M D-galactose showed that approximately 4.5% of the hemocytes had adhering AHRBC. Similarly, monolayers containing 0.1 M alpha-methyl D-galactoside showed approximately 6% of the hemocytes with adhering AHRBC. These represent a significant reduction ($p<0.05$) in the percent hemocytes with adhering erythrocytes as compared to DPBS incubated controls, which averaged 11% hemocytes with adhering AHRBC (Fig. 10). Slight decreases in the mean number of erythrocytes per hemocyte were also observed upon carbohydrate incubation. DPBS incubated control hemocytes that have adhering AHRBC have on average 10 AHRBC per hemocyte, while D-galactose and alpha-methyl-D-galactose incubated monolayers have approximately 8 and 7 adhering AHRBC, respectively (Fig. 11). This decrease was not statistically significant.

A wide variety of carbohydrates which inhibit the agglutination of asialo human erythrocytes in hemagglutination assays (46) were tested for inhibition of erythrocyte rosetting. Among the galactosidic carbohydrates melibiose, D-fucose, raffinose, 2-deoxy-D-galactose, alpha-methyl-D-galactose, and D-galactose (all 0.1 M), only alpha-methyl-D-galactose and D-galactose were inhibitory. Glucosidic carbohydrates tested and found to have no effect on erythrocyte rosetting were palatinose, maltotriose,

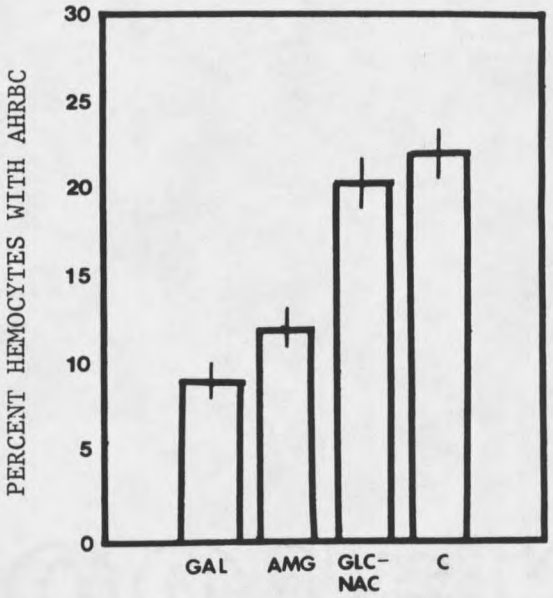

Figure 10. Effect of carbohydrates on the percent hemocytes with associated AHRBC. GAL-D-galactose, AMG-alpha-methyl-D-galactose, GLCNAC-N-acetyl glucosamine (all 0.1 M), C-control. Mean values ±1 S.E.

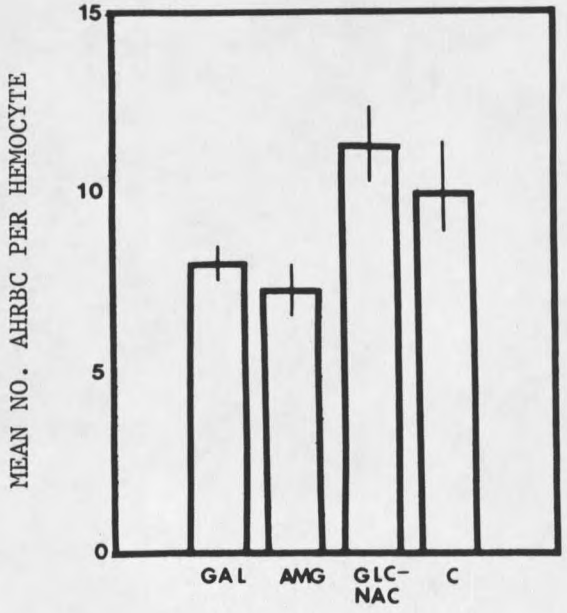

Figure 11. Effect of carbohydrates on the mean number of AHRBC associated with hemocytes. GAL-D-galactose, AMG-alpha-methyl-D-galactose, GLCNAC-N-acetylglucosamine (0.1 M), C-control. Mean values ±1 S.E.

melezitose, alpha-methyl-D-glucose, and D-glucose. The addition of N-acetylglucose amine, a non-inhibitor of hemagglutination, has no significant effect on erythrocyte rosetting as compared to DPBS incubated control hemocytes.

Of the above glucosidic and galactosidic carbohydrates, none were found to have any significant effect on the phagocytosis of Nosema spores or the adherence of B. thuringiensis bacteria. Under the conditions of this experiment, the binding of these two particles to hemocytes does not appear to be dependent on carbohydrates, indicating that membrane-bound hemaggluinin may not be mediating the hemocyte-particle interaction.

Particles Incubated in Hemolymph

Asialo Human Erythrocytes. Control AHRBC incubated in DPBS prior to overlayering monolayers adhered to approximately 22% of the monolayer hemocytes. Erythrocytes incubated in grasshopper serum at serum concentrations of 1:8, 1:32, and 1:256, then directly overlaid on monolayers without washing the AHRBC to remove hemolymph, adhered to approximately 8, 18, and 21%, respectively, of the hemocytes (Fig. 12). This decrease in hemocytic adherence is significant ($p<0.05$) for the higher concentrations of hemolymph, 1:8 and 1:32. Figure 13 shows that a significant increase in the mean number of AHRBC adhering to hemocytes occurred for AHRBC pretreated with 1:8 serum (5 AHRBC per hemocyte) as compared to DPBS incubated control erythrocytes (3 AHRBC

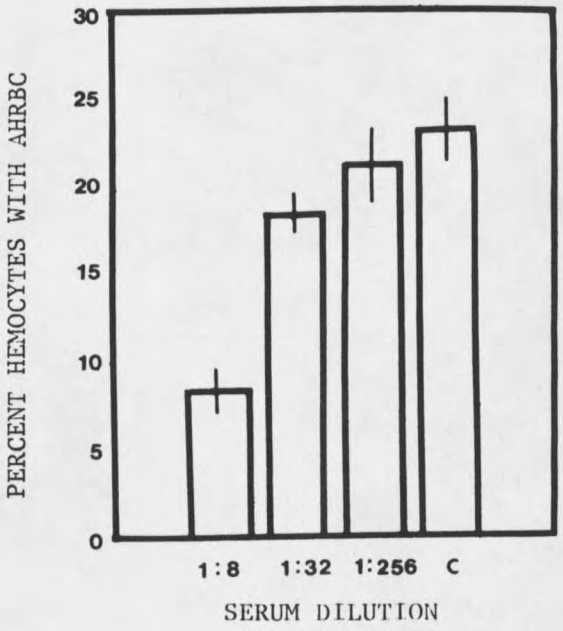

Figure 12. Percent hemocytes with associated serum-pretreated, unwashed AHRBC. C-DPBS control. Mean values ± 1 S.E.

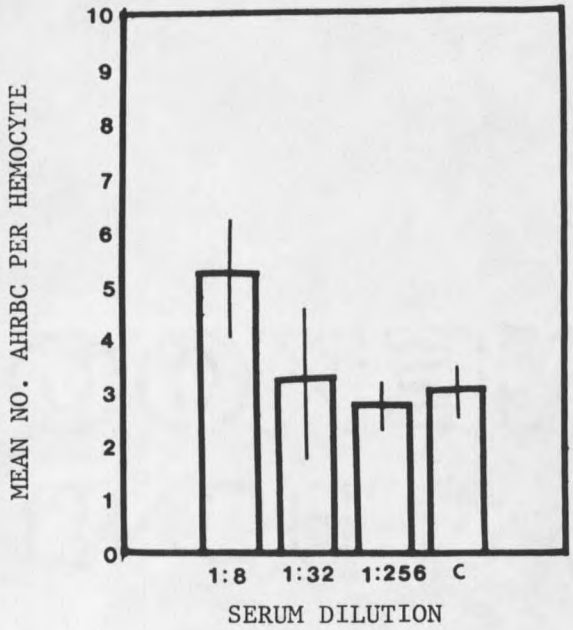

Figure 13. Mean number of serum-pretreated, unwashed, AHRBC associated with hemocytes. C-DPBS control. Mean values ± 1 S.E.

per hemocyte). The 1:32 and 1:256 dilutions of hemolymph used for pretreating erythrocytes showed no significant effect on adherence to hemocytes as compared to controls.

Monolayers incubated in serum-pretreated and washed AHRBC also showed a significant decrease in AHRBC adherence at the 1:8 hemolymph dilution (11% of the hemocytes) as compared to DPBS-incubated hemocytes (18% of the hemocytes). No significant difference in the percent hemocytes with adhering AHRBC was observed for the 1:32 and 1:256 serum dilutions (Fig 14). The mean number of erythrocytes adhering to hemocytes showed a slight increase over control values for the 1:8 serum incubated concentration (3.6 versus 3.0 adhering AHRBC), however this was not a significat difference. The higher dilutions of serum, 1:32 and 1:256 alsoshowed no significant difference in the mean number of AHRBC per hemocyte with adhering erythrocytes as compared to DPBS-incubated control AHRBC (Fig. 15).

In general, serum-pretreated AHRBC adhered to fewer numbers of monolayer hemocytes. This decrease in percent hemocytes with adhering AHRBC may be due to erythrocyte clumping. Red blood cells incubated in M. differentialis serum at the 1:8 dilutions showed significant clumping prior to overlaying the monolayers. To disrupt clumps, all erythrocyte solutions were then ejected through a 23 gauge syringe needle prior to adding to the monolayer. However, agglutination was still observed on the monolayers at the

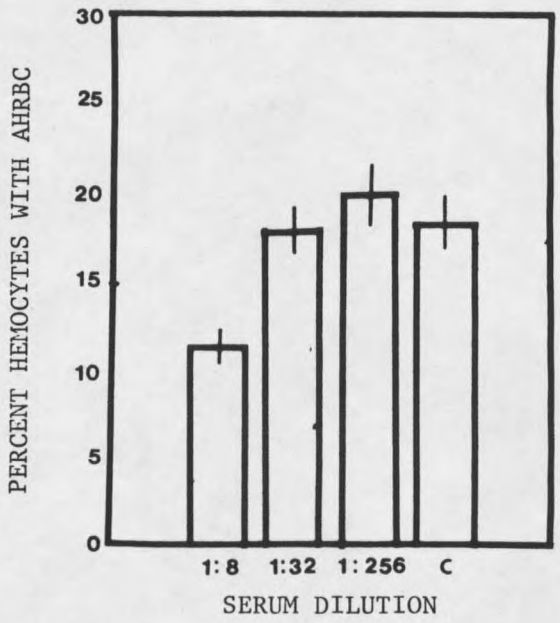

Figure 14. Percent hemocytes with associated serum-pretreated, washed, AHRBC. C-DPBS control. Mean values ± 1 S.E.

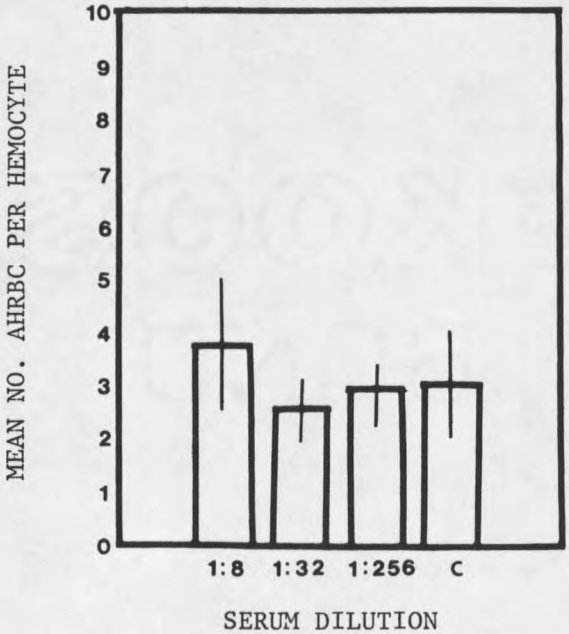

Figure 15. Mean number of serum-pretreated, washed AHRBC associated with hemocytes. C-DPBS control. Mean values ± 1 S.E.

1:8 dilutions. Erythrocyte clumping is also the probable reason for the increased mean number of adhering AHRBC per hemocyte seen at the 1:8 serum incubations.

Nosema locustae spores. Grasshopper hemocytes overlaid with *Nosema* spores preincubated soley in DPBS exhibited approximately 6.5% of the hemocytes with engulfed spores. Spores preincubated in *M. differentialis* serum, then added to monolayers unwashed, showed no significant difference in the percent cells that phagocytized one or more spores at any of the hemolymph concentrations (Fig 16). However, the mean number of *Nosema* engulfed per phagocytic cell (plasmatocyte) was affected by hemolymph pretreatment. Plasmatocytes overlaid with 1:8, 1:32, and 1:256 serum-pretreated, unwashed, spores phagocytized approximately 9.5, 5, and 6 spores, respectively. This is compared to control, DPBS-incubated spores which were phagocytized on average of 15.5 spores per plasmatocyte (Fig. 17). This decrease is significant only for the 1:32 and 1:256 preincubated spores.

Nosema incubated in grasshopper serum dilutions and washed prior to overlaying monolayers showed a general increase in the percent hemocytes that had adhering or phagocytized spores. Figure 18 shows that 1:8 and 1:32 serum pretreated and washed spores are associated with 14.5 and 13.5% of the hemocytes, respectively. This is a significant increase over control, DPBS-pretreated,washed, spores, which have *Nosema* associated with only 7% of the

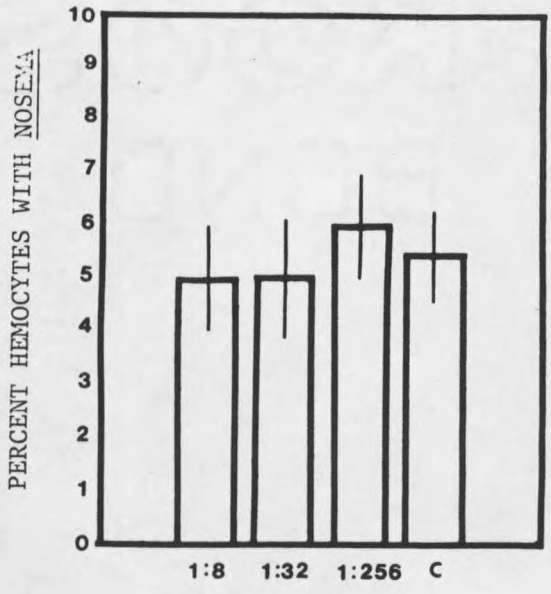

Figure 16. Percent hemocytes with associated serum-pretreated, unwashed, Nosema locustae spores. C-DPBS contol. Mean values ± 1 S.E.

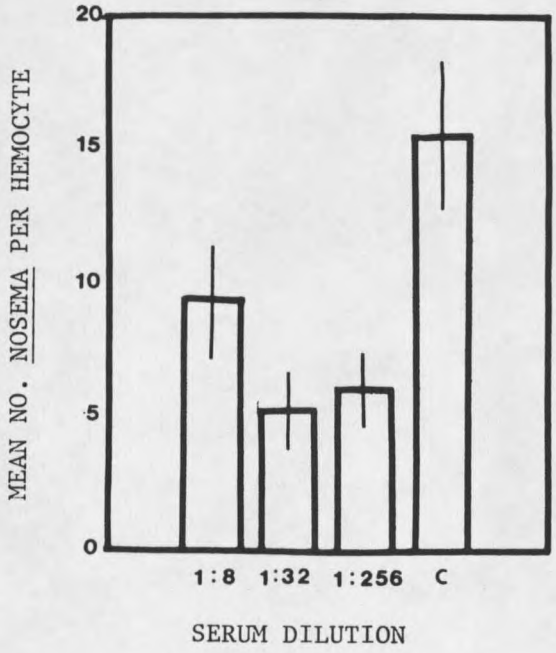

Figure 17. Mean number of serum-pretreated, unwashed, Nosema locustae spores associated with hemocytes. C-DPBS contol. Mean values ± 1 S.E.

cells. No significant difference in hemocyte associated spores was seen for the 1:256 serum dilution of pretreated spores (8%) as compared to the controls. Hemocytes overlaid with serum-pretreated, washed, spores had on average 3.5-5 spores each (Fig. 19). This represents no significant difference from the DPBS-incubated and washed controls.

Hemolymph pretreatment of Nosema spores led to a general increase in spore stickiness. Hemolymph pretreated spores were observed to adhere to the monolayer culture dish, as well as to plasmatocytes and granulocytes. In contrast, non-pretreated Nosema spores are associated exclusively with plasmatocytes. This increase in adherence was especially noticeable for hemocytes overlaid with serum-pretreated and washed spores (Fig. 18). In addition, spores incubated in grasshopper serum were observed to clump slightly. These clumps generally involved few spores, and were difficult to avoid. Clumping of hemolymph incubated Nosema has been previously reported by Jurenka and coworkers (16).

Of the two particles tested, AHRBC and Nosema spores, grasshopper hemolymph caused an increased particle association with hemocytes only for serum-pretreated, washed, Nosema. Incubating AHRBC in grasshopper serum resulted in a general decrease in the percent hemocytes with adhering erythrocytes. However, the serum-pretreated and washed Nosema showed a significant increase in the percent

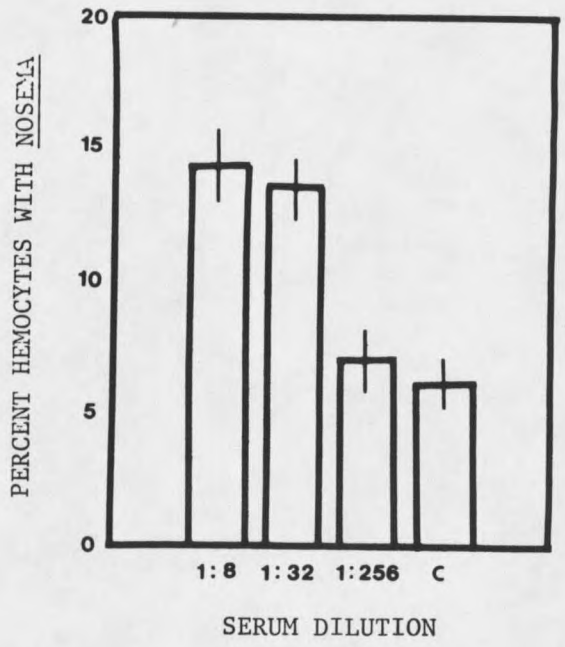

Figure 18. Percent hemocytes with associated serum-pretreated, washed, Nosema locustae spores. C-DPBS control. Mean values ± 1 S.E.

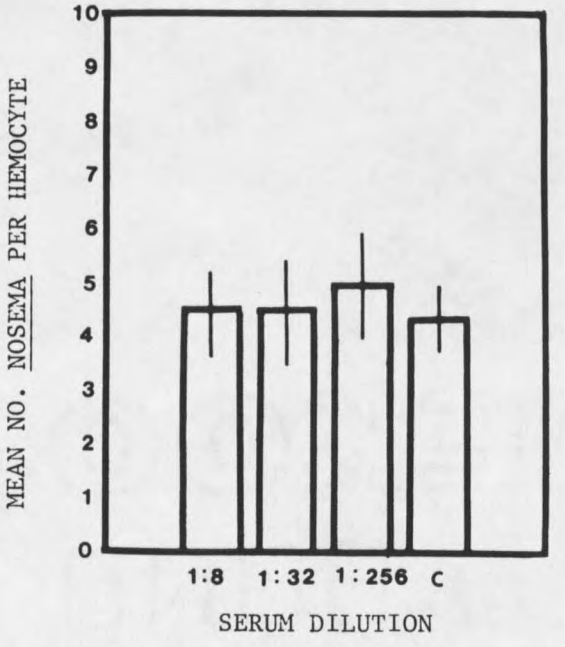

Figure 19. Mean number of serum-pretreated, washed, Nosema locustae spores associated with hemocytes. C-DPBS control. Mean values ± 1 S.E.

hemocytes with adhering or phagocytized spores. This increase in hemocyte-Nosema interaction may be the result of a hemolymph opsonin. Whether this possible opsonin is hemagglutinin must be tested by incubating particles in purified hemagglutinin.

Particles Incubated in Purified Agglutinin

Similar to the whole hemolymph studies, asialo human erythrocytes, Nosema spores, and B. thuringiensis bacteria were incubated in purified hemagglutinin at dilutions of 1:8, 1:32, and 1:256 in DPBS. This represents approximate agglutinin concentrations of 20, 5, and 0.5 ug/ml, respectively.

Erythrocyte Rosetting. Monolayers overlaid with agglutinin-pretreated and unwashed erythrocytes showed a general decrease in the percent hemocytes that had adhering RBCs. Erythrocytes pretreated with 20, 5, and 0.5 ug/ml agglutinin adhered to 4.5, 14.5, and 31% of the hemocytes, respectively. Control AHRBC, preincubated soley in DPBS adhered to approximately 26.5% of the monolayer hemocytes (Fig. 20). These differences from the control values are significant for erythrocytes incubated in 20 and 5 ug/ml concentrations of agglutinin. The slight increase in the percent hemocytes with adhering AHRBC seen for the 1:256 agglutinin-incubated erythrocytes was found not to be significant. The mean number of HRBC adhering to hemocytes

showed a significant increase for the 20 ug/ml agglutinin dilution (6.5 AHRBC per hemocyte), while no significant difference was observed for the 5 and 0.5 ug/ml agglutinin pretreated AHRBC as compared to control monolayers, which had on average 3 AHRBC per hemocyte. (Fig 21).

The percent adherence of AHRBC to hemocytes overlaid with agglutinin pretreated and washed erythrocytes also showed a significant decrease for the 20 and 5 ug/ml dilutions of agglutinin (7 and 19%), as compared to control hemocytes (34%) (Fig. 22). No significant difference was observed in the mean number of HRBC adhering to hemocytes among all the agglutinin concentrations as compared to controls (Fig. 23).

In general, agglutinin pretreatment of asialo human erythrocytes resulted in their decreased adherence to hemocytes. The erythrocytes were seen to clump at the higher concentrations of agglutinin, 20 and 5 ug/ml. This is probably the reason for the lower percentage adherence seen at these concentrations, and for the increased mean number of AHRBC per hemocyte with adhering erythrocytes observed at the 20 ug/ml agglutinin dilution.

Nosema spores. Hemocytes overlaid with DPBS-incubated spores have on average 6% of the hemocytes with associated spores. Almost exclusively this represents plasmatocytes that have phagocytized Nosema. No significant differences were observed in the percent phagocytosis by hemocytes

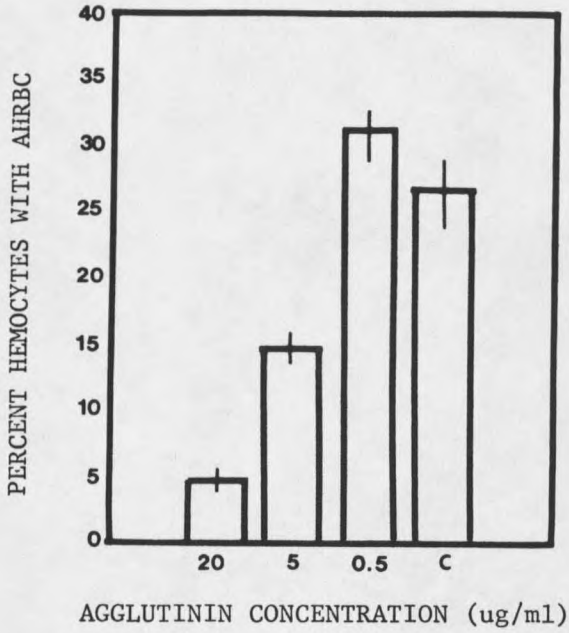

Figure 20. Percent hemocytes with associated agglutinin-pretreated, unwashed AHRBC. C-DPBS control. Mean values ± 1 S.E.

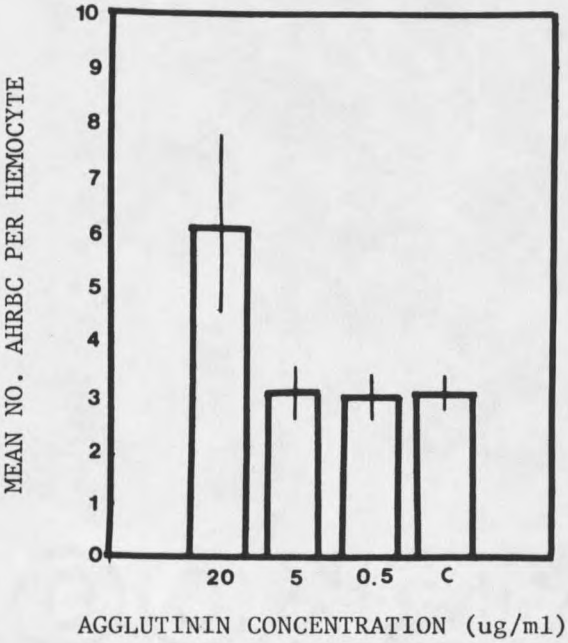

Figure 21. Mean number of agglutinin-pretreated, unwashed AHRBC associated with hemocytes. C-DPBS control. Mean values ± 1 S.E.

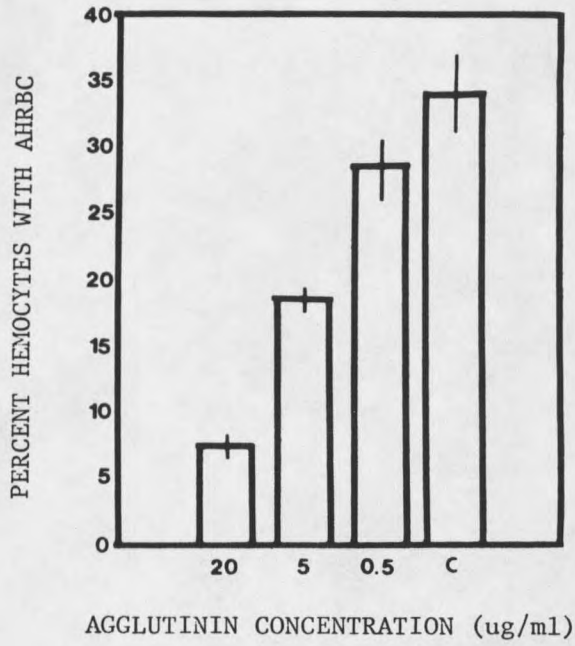

Figure 22. Percent hemocytes with associated agglutinin-pretreated, washed AHRBC. C-DPBS control. Mean values ± 1 S.E.

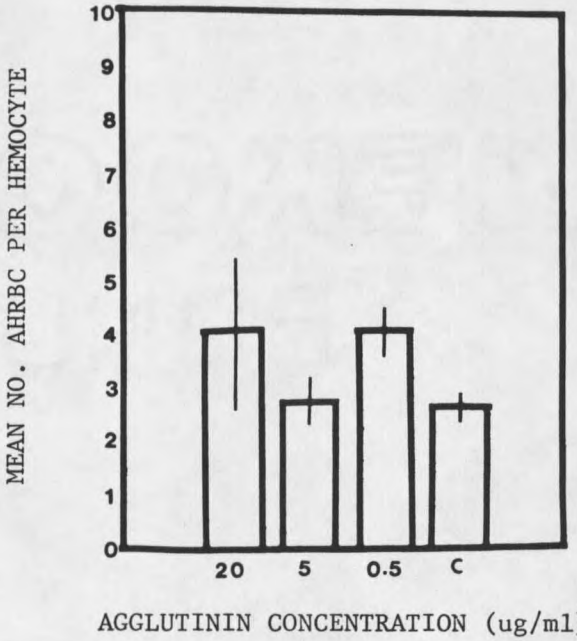

Figure 23. Mean number of agglutinin-pretreated, washed, AHRBC associated with hemocytes. C-DPBS control. Mean values ± 1 S.E.

overlaid with agglutinin-pretreated, unwashed, Nosema as compared to control, (DPBS-incubated) spores (Fig 24). Also, no significant difference was seen in the mean number of agglutinin-pretreated, unwashed, Nosema phagocytized by the plasmatocytes as compared to controls (Fig 25).

Monolayers overlaid with agglutinin-pretreated and washed spores also showed no significant difference in the percent cells that had phagocytized spores at all agglutinin concentrations as compared to the control value of 6% phagocytosis (Fig 26). Comparable to the aggutinin-pretreated and unwashed spores, washed spores also showed no significant difference in the mean number of spores engulfed per phagocytic cell (approximately 3-5 spores per plasmatocyte) (Fig. 27).

B. thuringiensis. Figure 28 shows the results of incubating B. thuringiensis bacteria in pure agglutinin, followed by adding the unwashed bacteria to hemocytes. In general, a decrease in bacterial adherence to hemocytes is observed. On average, DPBS-incubated bacteria adhere to 75% of the monolayer hemocytes. Pretreated unwashed bacteria at agglutinin concentrations of 20, 5, and 0.5 mg/ml adhere to 55, 62, and 68% of the hemocytes, respectively. This decrease in adherence with increasing agglutinin concentration is significant for agglutinin concentrations of 20 and 5 ug/ml incubated bacteria. Similarly, the agglutinin-pretreated bacteria adhere to hemocytes in less

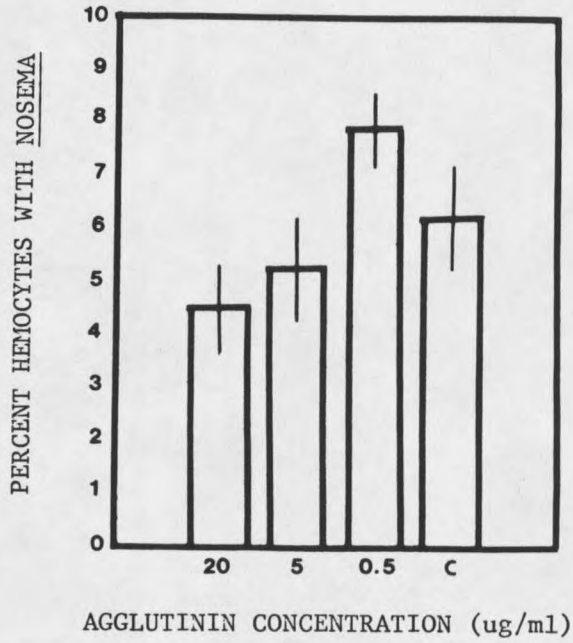

Figure 24. Percent hemocytes with associated agglutinin-pretreated, unwashed, Nosema locustae spores. C-DPBS contol. Mean values ± 1 S.E.

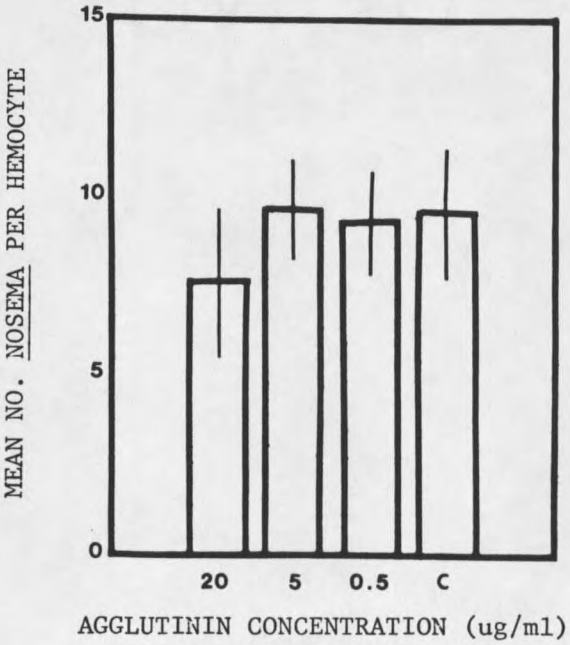

Figure 25. Mean number of agglutinin-pretreated, unwashed, Nosema locustae spores associated with hemocytes. C-DPBS control. Mean values ± 1 S.E.

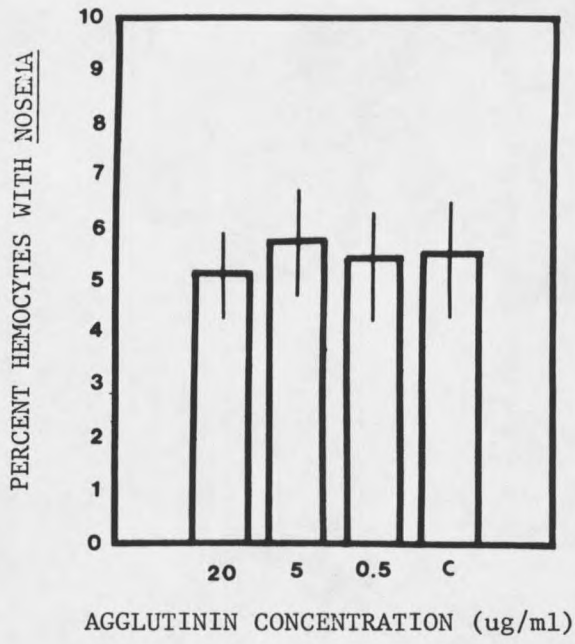

Figure 26. Percent hemocytes with associated agglutinin-pretreated, washed, Nosema locustae spores. C-DPBS control. Mean values ± 1 S.E.

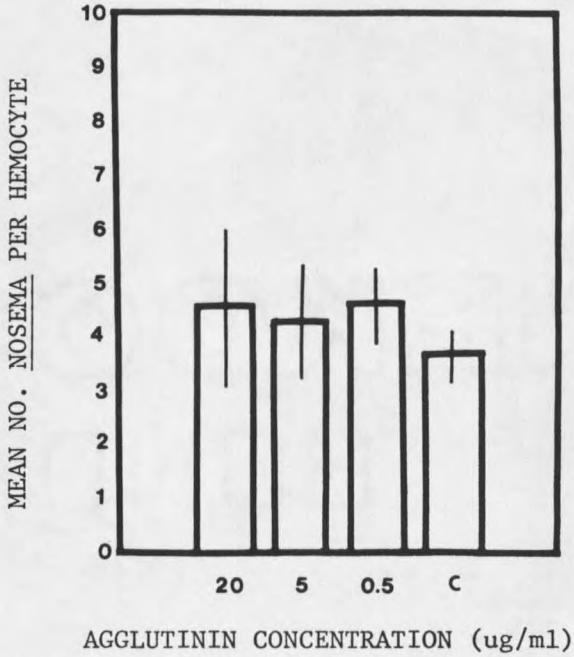

Figure 27. Mean number of agglutinin-pretreated, washed, Nosema locustae spores associated with hemocytes. C-DPBS control. Mean values ± 1 S.E.

numbers. The mean number of adhering bacteria per hemocyte with one or more bacteria decreases from the control value of 3.2 to 2.8, 2.4, and 2.3 for the 0.5, 5, and 20 ug/ml agglutinin pretreated bacteria. This decrease is significant for bacteria incubated in the highest concentration of agglutinin, 20 ug/ml as compared to control (DPBS-incubated) bacteria (Fig. 29).

When the agglutinin-pretreated bacteria were washed after incubation, to remove nonadhering hemolymph components, the percent hemocytes that have adhering bacteria also showed a decrease with increasing agglutinin concentration. Control, DPBS-incubated bacteria adhere to approximately 62% of the hemocytes, while the 0.5, 5, and 20 ug/ml agglutinin-incubated bacteria adhered to 51 50, and 38% of the hemocytes, respectively (Fig. 30). This decrease is significant only for the 20 ug/ml agglutinin-pretreatment. Control hemocytes which had adhering bacteria had on average 2.8 bacteria per cell. No significant difference in the mean number of bacteria adhering to hemocytes was found for agglutinin-incubated and washed bacteria (Fig. 31).

Bacteria incubated in hemagglutinin were not observed to agglutinate. Thus the decreases in the percentage adherence to hemocytes of agglutinin-pretreated bacteria cannot be attributed to clumping. Agglutinin-pretreatment of bacteria caused the bacteria to be less adherent to hemocytes.

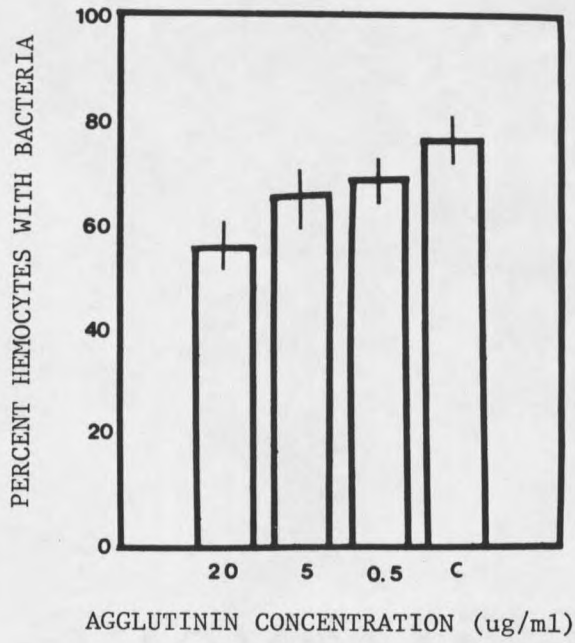

Figure 28. Percent hemocytes with associated agglutinin-pretreated, unwashed Bacillus thuringiensis bacteria. C-DPBS control. Mean values ± 1 S.E.

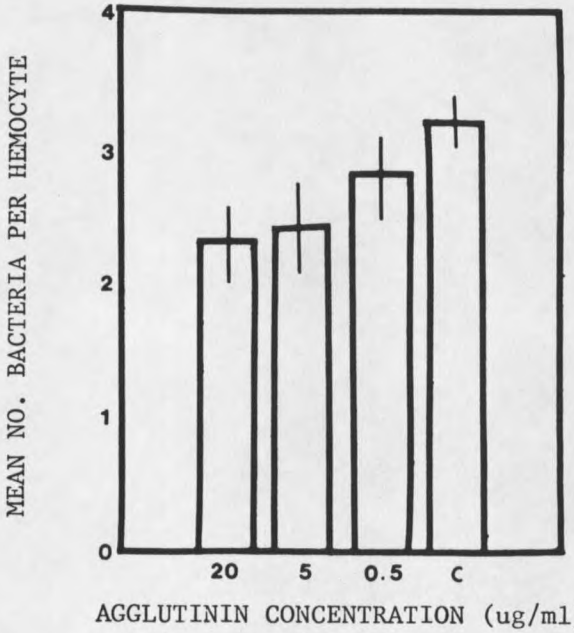

Figure 29. Mean number of agglutinin-pretreated, unwashed, Bacillus thuringiensis bacteria associated with hemocytes. C-control. Mean values ± 1 S.E.

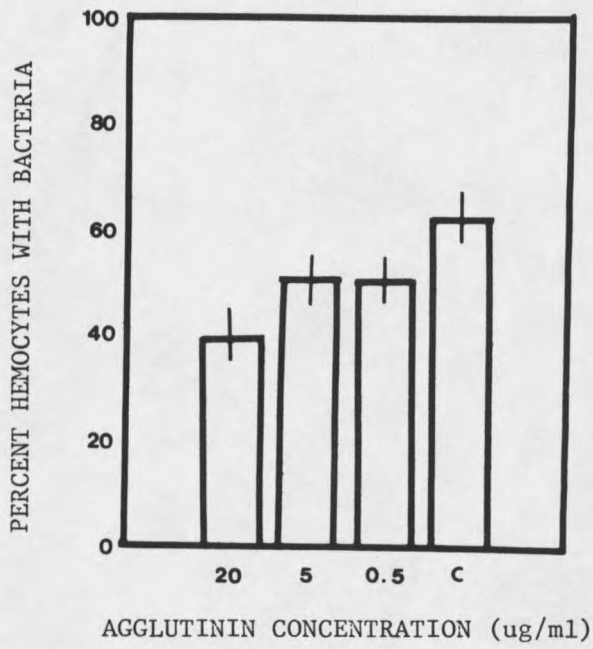

Figure 30. Percent hemocytes with associated agglutinin-pretreated, washed Bacillus thuringiensis bacteria. C-DPBS control. Mean values ± 1 S.E.

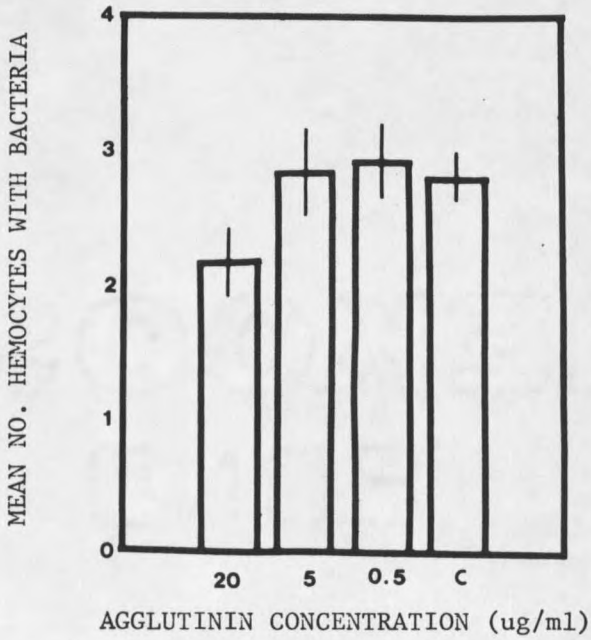

Figure 31. Mean number of agglutinin-pretreated, washed, Bacillus thuringiensis bacteria associated with hemocytes. C-control. Mean values ± 1 S.E.

This is most apparent at the highest concentrations of agglutinin (20 ug/ml) used for pretreating bacteria.

No increase in adherence or phagocytosis was observed when the tree test particles, AHRBC, Nosema spores and B. thuringiensis bacteria were preincubated in purified hemagglutinin prior to overlayering monolayer hemocytes. This indicates that purified hemagglutinin in vitro does not exhibit opsonic activity.

Hemocytes Incubated in Purified Hemagglutinin

Adherence/Phagocytosis. Monolayer hemocytes incubated in purified hemagglutinin (approximately 5 ug/ml), and overlaid with asialo human erythrocytes have approximately 7.5% hemocytes with adhering AHRBC. This percentage is not statistically different from control hemocytes incubated in DPBS prior to AHRBC overlayering (Fig. 32). Agglutinin pretreated monolayers show an average of 9 AHRBC per hemocyte with adhering erythrocytes. This was identical to DPBS-incubated control monolayers (Fig. 33).

Hemocytes incubated in purified hemagglutinin prior to overlayering with Nosema spores also showed identical percentages of hemocytes with engulfed spores (4.5%) (Fig. 34). The mean number of spores engulfed by plasmatocytes does not significantly differ between the agglutinin-incubated monolayers (10 spores) and the DPBS-incubated controls (10.5 spores) (Fig. 35).

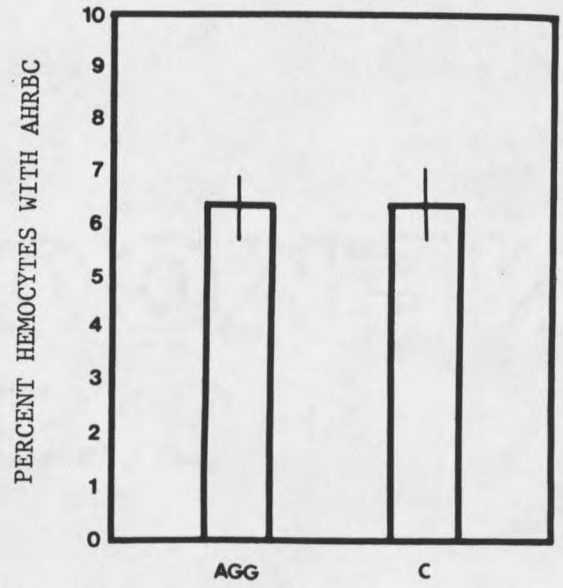

Figure 32. Effect of incubating monolayers in agglutinin (AGG) on percent hemocytes with associated AHRBC. C-DPBS control. Mean values ± 1 S.E.

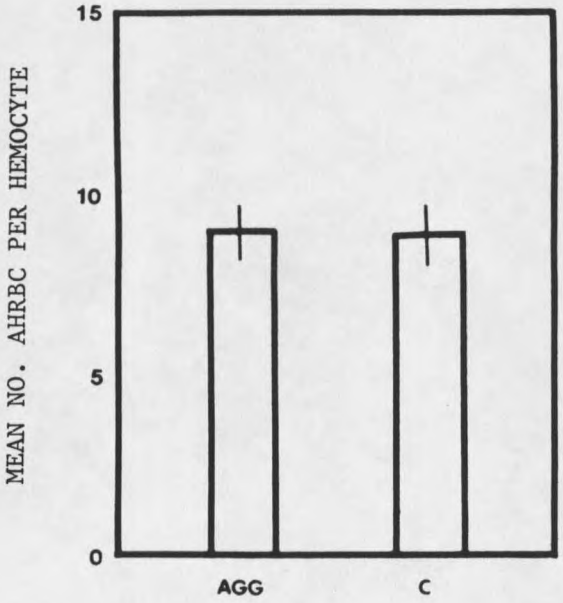

Figure 33. Effect of incubating monolayers in agglutinin (AGG) on the mean number of AHRBC associated with hemocytes. C-control. Mean values ± 1 S.E.

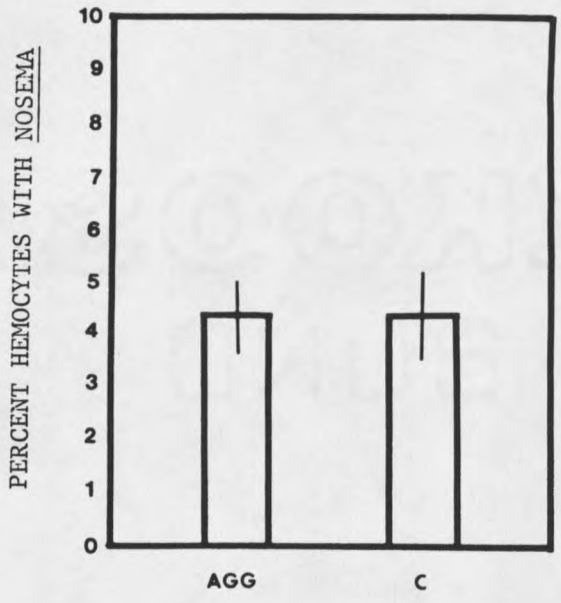

Figure 34. Effect of incubating monolayers in agglutinin on percent hemocytes with associated Nosema locustae spores. C-DPBS control. Mean values ± 1 S.E.

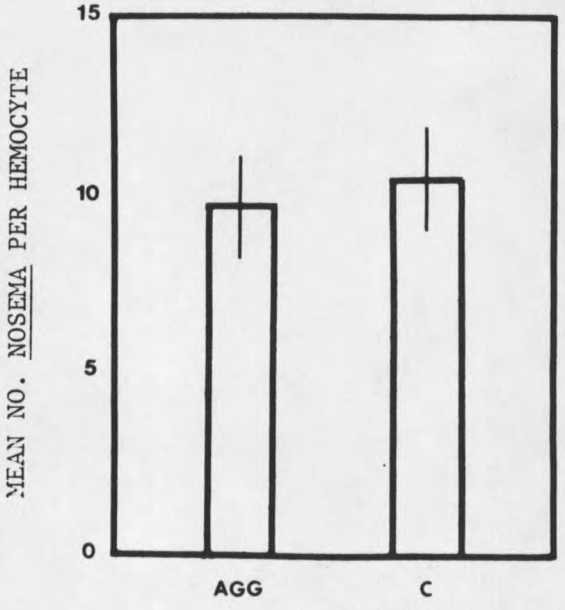

Figure 35. Effect of incubating monolayers in agglutinin on mean number of Nosema locustae spores associated with hemocytes. C-control. Mean values ± 1 S.E.

Immunostain. Live monolayered hemocytes incubated in purified agglutinin prior to fixing did not show any increase fluorescence over control cells incubated soley in DPBS and immunostained. This again indicated that agglutinin does not bind to hemocytic receptors *in vitro*.

The results of agglutinin-preincubated hemocyte experiments demonstrate that agglutinin did not bind to hemocytes, as assayed by increased particle adherence or increased immunofluorescent staining. This may indicate that agglutinin does not bind to hemocytic receptors and then bind or crosslink foreign particles, *in vitro*.

Blocking of Phagocytosis with Antibody.

Hemocytes incubated in the agglutinin-specific monoclonal antibody do not show a decrease in the percent cells that phagocytize *Nosema* spores. In fact, a significant increase is observed in the percentage hemocytes that phagocytize spores (17%) as compared to the naive mouse serum incubated control cells (12%) (Fig 36). The mean number of *Nosema* engulfed by phagocytic hemocytes did not significantly differ between antibody incubated (5.5 spores per phagocyte) and control monolayers (7.5 spores per phagocyte) (Fig. 37).

A significant lysing of the non-phagocytic granulocytes was observed when live hemocytes were incubated in the monoclonal antibody. This was not seen with the control, mouse serum incubated, cells. This lysis contibuted to the

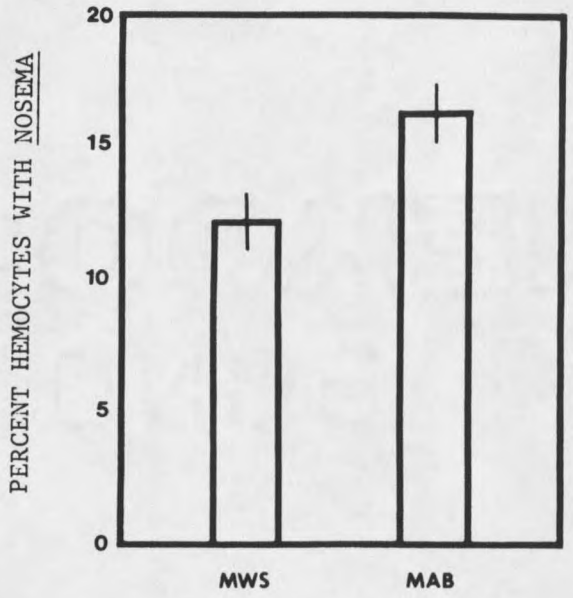

Figure 36. Effect of MAB on percent monolayer hemocytes with associated Nosema locustae spores. MWS-mouse whole serum. Mean values ± 1 S.E.

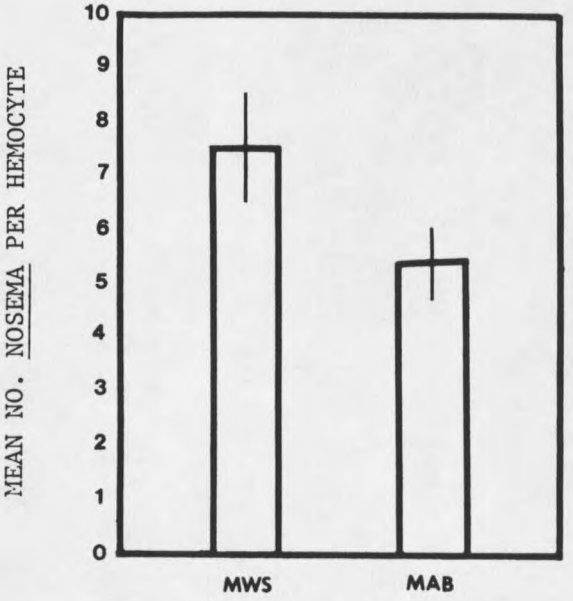

Figure 37. Effect of MAB on the mean number of Nosema locustae spores associated with hemocytes. MWS-mouse whole serum. Mean values ± 1 S.E.

higher percentage phagocytosis seen in the MAB-incubated monolayers, as more phagocytic cells were counted per total number of monolayered hemocytes.

The MAB was unable to block phagocytosis of *Nosema*, indicating that *in vitro* phagocytosis of *Nosema* is not agglutinin dependent. This correlates with the previous immunostain results that suggested the phagocytic cells lacked agglutinin, and the opsonization experiments that indicated a non-opsonic role for purified hemagglutinin towards *Nosema* spores, or any other particle tested.

DISCUSSION

Previous work in this laboratory has described the hemagglutinating activity present in the hemolymph of several grasshopper species, and has demonstrated that a single hemagglutinin protein accounts for all the hemagglutination activity found in Melanoplus differentialis and M. sanguinipes hemolymph (18,19,46). The data presented in this work establishes the presence of grasshopper hemagglutinin in adult hemocytes of M. differentialis and M. sanguinipes. The non-opsonic character of purified hemagglutinin is described and compared to similar results in other insect species.

Purification

This work employed a modification of the previously reported method to isolate grasshopper hemagglutinin by affinity chromatography on galactose-Sepharose (46). This modification incorporated a more extensive column wash using HEPES buffer, and column elution with alpha-methyl-D-galactoside, rather than D-galactose. Alpha-methyl-D-galactoside has been shown to be a more effective inhibitor of erythrocyte hemagglutination than is D-galactose (46), therefore it was thought to be a better competitor for

hemagglutinin binding sites. This would then result in higher yield protein elution from the Sepharose-galactose column. The use of the glycosidic derivative (a non-reducing carbohydrate) also reduces risk of spontaneous glycosylation of the protein. In combination with more extensive column washes with HEPES buffer, and increased time of incubation in the competing sugar, these modifications resulted in higher protein yields. The previous method typically resulted in approximately 350 ug purified protein (46) while in the new procedure 600-700 ug of protein is isolated from comparable amounts of hemolymph. Some reservations in this protein determination exist however, as some agglutinin preparations believed to contain protein, based on the modified Lowry assay, could not be shown to contain detectable agglutinin on silver stained gels. It is possible that this protein determination may be artificially high.

A modified Western blot-Con A probe (Fig. 2) showed that agglutinin purified by the previously reported technique may contain up to six contaminating glycoproteins that copurify with hemagglutinin, while agglutinin isolated by this modified technique showed no Con A sensitive bands, indicating the absence of these contaminating glycoproteins. These contaminants likely represent major hemolymph proteins that were incompletely washed from the column.

In addition to the Con A probe described above, the purity of the isolated hemagglutinin was analyzed by SDS-polyacrylamide gel electrophoresis followed by silver stain of the gel. One major protein band was detected in the gels, establishing the high purity of the hemagglutinin preparation (Fig. 3). Thus, the modifications in the affinity purification of hemagglutinin resulted in both higher protein yields and substantially greater purity.

Highly pure hemagglutinin, prepared as described above, was used for subsequent opsonization experiments, and represents a technical improvement over published reports where whole hemolymph rather than purified agglutinin had been used in opsonization experiments (43,44,45).

Purified insect agglutinins have been prepared by affinity chromatography in other insect species, including the flesh-fly Sarcophaga (21) and the beet armyworm Spodoptera (29). Komano and coworkers' (21) isolated a purified flesh-fly hemagglutinin which was shown to contain only a few minor protein contaminants when analyzed by SDS-PAGE followed by Coomassie blue staining of the gel. Pendland and Boucias's (29) affinity purified preparation of armyworm hemagglutinin appeared to be free from contaminating proteins when analyzed by SDS-PAGE followed by Coomassie blue gel stain. The purification of grasshopper hemagglutinin presented here is comparable to the above studies, as grasshopper hemagglutinin has been shown by SDS-

PAGE followed by silver staining to be free from protein contaminants. However, silver stains are considered to be 100-fold more sensitive for detecting trace amounts of proteins than are Coomassie blue stains (53). Thus a more rigorous demonstration of the purity of the isolated grasshopper hemagglutinin has been accomplished.

Localization

Monolayers used for the localization experiments consisted of hemocytes bled from adult or fifth instar nymphs. These grasshoppers were generally healthy, and resulted in intact, morphologically distinct, hemocytes. In comparison, use of old or diseased grasshoppers often resulted in monolayered hemocytes that easily burst or were extensively vacuolated. Use of these insects was avoided.

In general, the grasshopper monolayer hemocytes used for localization and opsonization studies are comparable to monolayer hemocytes use in other insectan studies (27,37,44, 45). However, direct comparisons between insect hemocytes are difficult, as insect hemocyte types vary greatly among species, and continuing controversy exists over the origin and classification of insectan hemocytes. For example, Amirante (37) identified four monolayer hemocyte types in the cockroach: plasmatocytes, granular cells, spherule cells, and coagulocytes; and Yeaton (27) identified six monolayer hemocyte types in the silkmoth: prohemocytes,

plasmatocytes, granulocytes, spherule cells, oenocytes, and adipohemocytes. These classifications are based on hemocyte morphology and ultrastructure (see Gupta, 56). In contrast, grasshoppers exhibited only two types of hemocytes recognizable on monolayers: granulocytes and plasmatocytes. Granulocytes constituted approximately 95% of the monolayer cells, while plasmatocytes consisted of 4-8% of the total monolayer population. However, an ultrastructural study of grasshopper hemocytes was not attempted, therefore a conclusive characterization has not been established.

Grasshopper hemagglutinin has been localized to hemocytes using three different immunochemical techniques. Western blotting of hemocyte lysates, indirect immunofluorescence, and indirect enzyme immunoassay have all detected the presence of agglutinin in monolayer hemocytes. Although Western blotting was useful as confirmation of the presence of agglutinin in hemocytes, it could not provide any information concerning the types of hemocytes that contain agglutinin, as could the immunocytochemical techniques. It is important to note that Western blots, using monoclonal antibody, of hemocyte lysates did show that the hemagglutinin present in hemocytes has the same electrophoretic pattern as the hemagglutinin found in grasshopper serum, suggesting that they are identical or closely similar molecules.

The presence of hemagglutinin in grasshopper hemocytes was confirmed by immunocytochemistry. Indirect immunofluorescence on both fixed monolayer and suspended cells, and indirect EIA all demonstrated hemocyte associated hemagglutinin. Indirect EIA staining did appear to be slightly more sensitive than was immunofluorescence (Fig. 9). This can be attributed to the enzymatic reaction that leads to tissue staining. The reaction between horseradish peroxidase and its substrate hydrogen peroxide and the electron donor DAB results in the formation of large opaque, insoluble DAB polymers (57). These polymers turn the labeled cells dark brown, while background staining remains minimal. Brown hemocytes are difficult to photograph in black and white, however, and due to the carcinogenic nature of DAB, indirect immunofluorescence remains the method of choice for localizing hemagglutinin in our lab.

Both immunofluorescence and EIA revealed that not all hemocyte types stain for the presence of agglutinin. In all cases, only the granular cells exhibited the agglutinin label, while plasmatocyte did not stain above background levels. In addition, only about 22-30% of total granulocytes specifically stained for the agglutinin. Some granular cells stained very intensely, other cells stained lightly, and others not at all. This suggests a variability in hemagglutinin content among granular cells. Whether or

not this may relate to subpopulations of granular cells is presently unknown.

Granular cells that stained for agglutinin by immunofluorescence and EIA techniques always exhibited cytoplasmic labeling. Label at or around the hemocyte plasma membrane was not observed. This may indicate that hemagglutinin is not membrane-bound. However, a more definitive test of membrane-bound antigen is to immuno-label live cells. Live, intact cells, kept at 4°C to prevent internalization, are impermeable to the antibody solutions, thus any fluorescent labeling observed could be attributed to outer membrane-bound agglutinin. When live monolayer hemocytes were subjected to an immunofluorescence protocol, no definitive results were obtained. For reasons not understood, a large number of granular cells were lysed by the MAB supernatant solution. This may in fact be due to the binding of antibodies to the hemocyte membranes, resulting in instability and a selective loss of hemagglutinin-containing cells. Since the antibody solutions were heat treated, complement activity is probably not responsible for the lysing. It is also possible that the lack of definitive immuno-staining was due to the extreme specificity of the MAB. As monoclonals are directed against a single antigenic determinant, the agglutinin, if present in the plasma membrane, may have had the epitope unavailable for recognition by the antibody.

It was hoped that the use of rabbit polyclonal antibodies would address the possible problem of MAB over-specificity. The rabbit antibodies, while specific for grasshopper hemagglutinin, presumably recognize several different agglutinin epitopes. However, rabbit antibodies proved unsuitable for labelling grasshopper hemocytes due to high nonspecific binding. It appears that grasshopper hemocytes may have membrane proteins that bind rabbit immunoglobulins. In the future, the question of membrane bound agglutinin may best be addressed by immunochemical localization at the electron microscope level.

Although hemagglutinin was not found to be associated with the hemocyte plasma membrane by immunocytofluorescence, it was thought that hemagglutinin receptors may exist on hemocytes, which function *in vivo* to bind agglutinin. Live monolayer hemocytes were incubated in purified hemagglutinin in an experiment designed to test for the presence of these hemocytic agglutinin receptors. Subsequent to agglutinin treatment, the monolayers were fixed and subjected to the immunocytofluorescence procedures. No increase in fluorescent staining over DPBS-incubated control monolayers was observed. In addition, agglutinin-treated monolayers did not bind AHRBC or phagocytize *Nosema* spores significantly different from the control hemocytes. Thus hemocytic agglutinin receptors were not detected, indicating that at least *in vitro* agglutinin does not bind to hemocytes.

Localization of hemagglutinin in grasshopper hemocytes can be compared to earlier work done on other insect species. Amirante (37), utilizing immunofluorescence techniques, localized cockroach agglutinin in the granulocytes and spherule cells from Leucophaea. Based on the staining patterns of fixed cells (staining of live cells was not reported), he determined agglutinin to be both cytoplasmic and membrane-bound. In his study, plasmatocytes did not label for the agglutinin. In a similar immunofluorescence study on the silkmoth Hyalophora, Yeaton (27) concluded that both plasmatocytes and granulocytes contained agglutinin, although granular cells were more intensely stained. Yeaton also concluded that agglutinin was both cytoplasmic and membrane-bound. Both of these studies utilized the immunofluorescence results to support additional research which concluded that hemagglutinin was synthesized by hemocytes, a question that was not addressed in this research, however other work in this laboratory suggests that it is not (47).

Both Amirante (37) and Yeaton (27) localized hemagglutinin using anti-agglutinin antisera prepared by injecting rabbits with hemolymph-agglutinated and washed rabbit erythrocytes. In her study of Periplaneta hemagglutinin, Lackie (38) utilized antibody prepared against purified hemagglutinin to demonstrate the presence of cockroach hemagglutinin in all hemocyte types. This

represents an improvement over Amirante's and Yeaton's work, as producing antibody against purified hemagglutinin should eliminate most hemolymph contaminants that might elicit antibody production. The work presented in this research differs significantly in this point as the antibodies used here were mouse monoclonals that have been demonstrated to be specific for grasshopper hemagglutinin. This technical improvement represents a much cleaner procedure for producing agglutinin-specific antibodies, and eliminates any possible antigenic contaminants that could lead to false positive staining.

In contrast to the lack of hemagglutinin receptors found in grasshopper hemocytes, Komano and coworkers (39) concluded that the flesh-fly Sarcophaga does contain hemocytic receptors for agglutinin. These receptor were demonstrated on the flesh-fly hemocyte outer membrane by their ability to bind radioiodinated flesh-fly lectin in vitro. Use of a radioiodinated agglutinin molecule represents a direct assay of hemagglutinin binding, whereas the methods employed in localizing grasshopper agglutinin receptors (indirect IFA and AHRBC or Nosema adherence) are indirect assays of hemagglutinin binding. These indirect methods may have been unsuited for detecting hemagglutinin receptors. It is possible that receptor-bound grasshopper hemagglutinin is no longer available for MAB recognition or in vitro particle binding. It is equally likely that hemagglutinin

that has bound to hemocytic receptors may then be dislodged by the additional manipulations involved in immunofluorescence or particle incubations. Although grasshopper hemocytic-agglutinin receptors have not been demonstrated in this study, more conclusive evidence of their presence or absence would involve use of directly labeled (either radioactive or fluorescent) agglutinin molecules.

To date, insect hemagglutinins have been demonstrated to be associated with the hemocytes of Orthopterans (cockroaches), a Lepidopteran (silkmoth), and a Dipteran (flesh-fly). The research presented here has extended hemocyte-associated hemagglutinin to one more Orthopteran, the grasshopper. Based on these studies it seems likely that hemocyte associated agglutinins will be found in more insect species, however the cell types that contain agglutinin may vary among species. Whether or not the presence of agglutinin may signify a functional subpopulation of hemocytes is an important but unanswered question.

In addition to being present in the hemocytes, hemagglutinin <u>in vivo</u> may also be localized to specific hemolymph glycoconjugates. In the hemolymph probing experiments described in this work, electrophoresed and blotted grassshopper hemolymph was incubated in additional grasshopper hemolymph to identify possible hemolymph receptors for hemagglutinin. The results of this modified Western blot indicated that at least five possible

hemagglutinin binding sites exist in grasshopper hemolymph (Fig. 4). These binding sites are presumably glycoproteins which bound to the agglutinin via carbohydrate-lectin interactions. A rigorous control to test this possible carbohydrate-lectin interaction would be to inhibit hemagglutinin binding with the addition of alpha-methyl-D-galactoside to the hemolymph probe. However, this was not performed. Therefore the agglutinin-specific nature of these bands is tenuous. However, if shown to be agglutinin-specific binding sites, they may represent glycoproteins that are in vivo binding substances for agglutinin. These molecules may be glycoproteins that are transported by hemagglutinin in vivo, or may be proteins that serve to modify hemagglutinin activity in vivo, as previously suggested by Stebbins and Hapner (46). Further work in this area will help to elucidate the function and identity of these hemolymphatic receptors.

Opsonic Characterization

Grasshopper hemocytes are capable of binding or phagocytizing a variety of test particles in vitro. While these particles may bind to both granulocytes and plasmatocytes, only plasmatocytes appear to be phagocytic In vitro. However, in vivo, granulocytes may have some phagocytic capacity. It is not uncommon to obtain a grasshopper that has a slight Malameba locustae infection.

Malameba cysts are seen in the fresh-bled hemolymph of these insects, and occassionally both granulocytes and plasmatocytes will have phagocytized one or more cysts. As evidenced by the washed monolayer hemocytes, phagocytosis and adherence can occur without the presence of any serum factors. Thus, humoral agglutinin is not a prerequisite for hemocyte activity.

Membrane-bound Hemagglutinin

The ability of insect hemocytes to form rosettes of vertebrate erythrocytes has often been taken as evidence of membrane-bound agglutinin on the hemocytes. Since hemagglutinins can agglutinate erythrocytes *in vitro*, it was inferred that erythrocyte rosetting was also a function of agglutinins. Grasshopper hemocytes are also observed to bind asialo human erythrocytes *in vitro*. Thus, grasshopper hemocytes may have membrane-bound agglutinin that functions in the adherence of AHRBC.

As a test for the presence of membrane-bound agglutinin, carbohydrates were added to the monolayers to inhibit rosette formation. These carbohydrates were chosen from known erythrocyte hemagglutination inhibitors (46). If membrane-bound agglutinin was responsible forerythrocyte rosetting, these carbohydrates should compete for the lectin binding sites and inhibit rosetting. Several glucosidic and galactosidic inhibitors of hemagglutination were tested. Among these only D-galactose and alpha-methyl-D-galactoside

inhibited erythrocyte rosetting, and this inhibition was not complete (Fig. 10). The fact that the other carbohydrate inhibitors of hemagglutination did not inhibit erythrocyte binding to hemocytes can be viewed as consistent with the immunochemical hemagglutinin localization results. As of yet, membrane-bound grasshopper hemagglutinin has not been detected on monolayer hemocytes by immunocytochemistry. Thus the binding of erythrocytes to grasshopper hemocytes may not be dependent on outer membrane-bound agglutinin.

Similar carbohydrate inhibition experiments were performed on hemocytes overlaid with Nosema spores or B. thuringiensis bacteria. In both of these experiments no carbohydrates were found which had any significant effect on adherence or phagocytosis. This provides another piece of evidence to indicate that membrane-bound agglutinin, if indeed present on hemocytes, is not responsible for the adherence of these two test particles.

In an additional experiment designed to test for the participation of outer membrane-bound agglutinin in hemocytic defense activities, blockage of in vitro hemocyte phagocytosis was attempted by incubating live hemocytes with Nosema in the presence of MAB. Phagocytosis was not blocked by the MAB, offering yet another piece of evidence that membrane-bound agglutinin does not mediate phagocytosis of Nosema. In fact, a significant increase in the percent hemocytes that phagocytized Nosema was detected for

monolayers incubated in MAB (Fig. 36), but this was due to the granular cell lysing that occurred in the presence of the MAB.

The results of this attempt to inhibit phagocytosis with MAB are consistent with the inability to detect plasmatocyte-associated hemagglutinin in immunofluorescence staining of monolayer hemocytes with MAB, and with the inability to inhibit phagocyosis with agglutinin-specific carbohydrates. As the phagocytic cells do not stain for the presence of agglutinin, it would be expected that agglutinin-specific antibodies would not block phagocytosis. Thus, membrane-bound agglutinin does not appear to be involved in the binding and phagocytosis of Nosema spores. The agglutinin-specific rabbit polyclonals were not used to block Nosema phagocytosis due to the high non-specific binding of rabbit immunoglobulins as reported for immunofluorescence work, and consequent uncertainty in experimental interpretation.

Agglutinin as a Humoral Opsonin

As a test for the role of grasshopper hemagglutinin as a potential opsonin, various test particles (AHRBC, Nosema spores, B. thuringiensis bacteria) were incubated in either grasshopper serum or purified hemagglutinin, and then overlaid on monolayer hemocytes. In some of these experiments the serum-preincubated particles were washed prior to overlayering monolayers. This was intended to remove all non-

adhering serum molecules, leaving only particle-bound proteins, or potential opsonins.

Under the conditions of this study, no opsonic capacity against human asialo erythrocytes was found for either grasshopper serum or purified hemaglutinin *in vitro*. In general, serum pretreatment of asialo human erythrocytes decreased the percent hemocytes with adhering AHRBC. This is most noticeable with serum treated, unwashed, AHRBC, but is significant for monolayers overlaid with both washed and unwashed pretreated erythrocytes (Figs. 12-15). Similarly, agglutinin-pretreated AHRBC showed a general decreased adherence to hemocytes as compared to DPBS-treated control AHRBC (Figs. 20-23). This decline in percent hemocytes with adhering serum- or agglutinin-pretreated AHRBC is probably due to erythrocyte agglutination, which is visually observable for AHRBC incubated in the higher hemolymph concentrations (1:8 and 1:32). Erythrocyte agglutination resulted in fewer numbers of individual erythrocytes, but larger sized erythrocyte clumps. AHRBC agglutination also appears to have contributed to the increased mean number of erythrocytes that bound to hemocytes, as the large AHRBC clumps tended to stick to hemocytes. The results of these experiments indicate that agglutinin does not exhibit opsonic activity towards AHRBC *in vitro*. In fact, no opsonic activity *in vitro* was detected for grasshopper serum.

In contrast to serum-pretreated AHRBC, a potential serum opsonin was found for Nosema spores. Nosema that were pretreated with serum, then overlayered on the monolayers after washing to remove nonadhering hemolymph components, did show an increase in the percent hemocytes with associated spores (Fig. 18). This increase in hemocyte-associated spores was not due to an increase in phagocytosis, rather a general increase in the adherence (stickiness) of the spores to both granulocytes, plasmatocytes, and the culture dish was observed. In addition, these spores were observed to clump slightly. Nontreated, or DPBS-incubated spores normally are seen associated with plasmatocytes exclusively; they do not adhere to granular cells or the culture dish, and they do not clump. This increase in hemocyte-associated spores was not observed when serum-pretreated Nosema were added to the monolayers without a DPBS wash (Fig. 16). In this case, the percent hemocytes with associated spores did not differ significantly from controls. This was probably due to Nosema agglutination, which may have been greater when the serum was not removed from the spores. This would result in large Nosema clumps which were then washed off the monolayer.

This increase in hemocyte-associated Nosema spores observed for monolayers incubated in serum-pretreated and washed spores suggests the presence of a serum opsonin that is responsible for coating spores, making them more easily

bound to hemocytes. Due to the complex molecular nature of grasshopper serum, the identity of this possible opsonin cannot be determined based on these experiments alone. However, in light of the agglutinin-pretreated Nosema experiments, this potential molecule is not likely to be hemagglutinin. Nosema spores, pretreated with purified grasshopper hemagglutinin, were found associated with hemocytes equally well as nontreated control spores (Figs. 24-27). Both agglutinin-pretreated washed and unwashed spores are found associated with hemocytes equally well as DPBS-treated control spores. In contrast to serum-treated spores, agglutinin-pretreated Nosema did not appear sticky. Agglutinin-pretreated spores were found associated soley with plasmatocytes, spores did not adhere to granulocytes or to the culture dish. In addition, agglutinin-treated spores did not clump. Thus it can be concluded that the serum component that caused an increase in the adherence of Nosema spores to hemocytes was probably not agglutinin, and Nosema does not bear GHA-specific receptors.

As a third biotic particle used to test for a possible opsonic role of grasshopper hemagglutinin, a bacterium, B. thuringiensis was incubated in purified agglutinin and overlaid on monolayer hemocytes. Consistent with the results for agglutinin-pretreated AHRBC and Nosema, monolayers overlaid with agglutinin-pretreated B. thuringiensis did not show an increase in hemocyte-

associated bacteria. As in the case of AHRBC pretreated with purified hemagglutinn, pretreatment of B. thuringiensis with purified grasshopper hemagglutinin actually decreased the percent hemocytes with adhering particles (Figs. 28-31). This decrease in adherence was observed whether or not the bacteria were washed prior to overlayering hemocytes. B. thuringiensis was not observed to clump upon agglutinin incubation. Thus the decrease in adherence of the bacteria to hemocytes cannot be attributed to agglutination. This suggests that agglutinin pretreated bacteria are actually less adherent to hemocytes. In such a case agglutinin may be coating the bacteria making it less recognizable for binding by the hemocytes. However, attempts to detect hemagglutinin by immunofluorecence labeling of the agglutinin-incubated bacteria were unsuccessful. This leaves open the question of why bacterial adherence actually decreased upon agglutinin-pretreatment.

A non-opsonic role for insect hemolymph has been demonstrated in previous studies with other insects. Anderson (43), incubated bacteria (Staphylococcus strains) in cockroach (Blaberus) serum, then added to the monolayers either washed to remove serum components or unwashed (still in serum). In all cases phagocytosis of the of the bacteria was not increased over control, untreated, bacteria. Scott (44), similarly pretreated chicken and sheep erythrocytes with cockroach (Periplaneta) serum prior to overlayering

cockroach hemocytes. No increase in erythrocyte adherence or phagocytosis was observed, rather the trend being towards a reduction in erythrocyte adherence with serum pretreatment. This observation was confirmed by Rowley and Ratcliffe (45), who also found no increase in phagocytosis by cockroach hemocytes when sheep erythrocytes were pretreated with Periplaneta serum. In addition, Rowley and Ratcliffe (45) also found similar results in a study of the stick insect Clitumnus. The percent Clitumnus hemocytes with associated erythrocytes decreased when the sheep erythrocytes were pretreated with stick insect serum. This was attributed to erythrocyte agglutination. Thus, to date no insect hemagglutinins or serum components have been described to show opsonic activity.

The results of the grasshopper serum-pretreated AHRBC experiments presented in this work are consistent with the above published reports for a non-opsonic activity of insect hemagglutinins. However, a potential serum opsonin that caused increased hemocytic Nosema adherence was found in this study, which has not been previously reported in the literature. The identity of this molecule is unknown, but it is not likely to be hemagglutinin. The results of the purified hemagglutinin-pretreatment experiments suggest that grasshopper hemagglutinin does not act as an opsonin. The use of purified grasshopper hemagglutinin represents a significant improvement over the previously published papers,

as to date, no purified insectan hemagglutinins have been reported to be used in opsonization studies, all studies having used insect serum for pretreating test particles. Thus, a more detailed test of the non-opsonic nature of insect hemagglutinins has been carried out by this research. However, further insectan studies will be needed before generalizations on the non-opsonic character of insect hemagglutinins can be made.

Although hemagglutinin has been detected in a percentage of the hemocytes in the grasshopper, it does not appear to have a role *in vitro* in the recognition of foreign particles by hemocytes. However, it cannot be concluded that grasshopper hemagglutinin plays no role in the insect's defense response. *In vivo*, grasshopper hemagglutinin may have some opsonic abilities. This will have to be tested by *in vivo* injections of various agglutinin-pretreated particles, followed by bleeding the grasshopper to examine the fate of the particles. Also, grasshopper hemagglutinin may serve a role in the defense response not specifically tested here. It is possible that hemagglutinin may function to bind foreign particles to the hemocytes responsible for encapsulation and nodule formation, or hemagglutinin may be involved in the degranulation response of granulocytes. Agglutinin *in vivo* may also serve bactericidal role, neutralizing or destroying potential pathogens before hemocytic responses are fully functioning. In addition, the hemolymph

probing experiments performed in this work may indicate a non-defense role for agglutinin in transport of glyco-conjugates.

CONCLUSIONS

This research has addressed the localization of grasshopper hemagglutinin in hemocytes, and some of the possible roles of hemagglutinin in the insect's defense system. In this regard, major conclusions to be drawn from the work are as follows:

1. Grasshopper hemagglutinin is present on the adult hemocytes of both *Melanoplus differentialis* and *M. sanguinipes*. Indirect immunofluorescence and indirect enzyme immunoassay labelling techniques have demonstrated hemagglutinin in the cytoplasm of approximately 25% of the granular hemocytes. The phagocytic hemocytes (plamatocytes) did not stain for the presence of agglutinin. Membrane-bound agglutinin was not detected in either granular cells or plasmatocytes.

2. Hemocytic membrane-bound agglutinin does not appear to be involved in the *in vitro* adherence or phagocytosis of asialo human erythrocytes, *Nosema* spores, or *B. thuringiensis* bacteria.

3. Grasshopper hemagglutinin does not exhibit opsonic activity towards asialo human erythrocytes, *Nosema* spores, and *B. thuringiensis* bacteria *in vitro*. Although a possible serum opsonin against *Nosema* spores was identified, this was

not attributed to hemagglutinin. In no case was affinity purified grasshopper hemagglutinin shown to cause an increase in the hemocytic adherence or phagocytosis of pretreated test particles.

4. The immunochemical localization of hemagglutinin in grasshopper hemocytes and the demonstration of the non-opsonic nature of grasshopper hemagglutinin *in vitro* are consistent with relevent published reports involving hemagglutinins from other insectan species.

REFERENCES CITED

1. Ratcliffe, N.A. 1985. Invertebrate immunity-a primer for the non-specialist. Immunol. Letters. 10:253-270.

2. Whitcomb, R.F., M. Shapiro, and R.R. Granados. 1978. Insect defense mechanisms against microorganisms and parasitoids, pp. 447-536. In M. Rockstein (ed.) The Physiology of Insecta, Vol. 5. Academic Press, Inc., New York.

3. Lackie, A.M. 1981. Immune recognition in insects. Dev. Comp. Immunol. 5:191-204.

4. Bang, R.B. 1975. Phagocytosis in invertebrates, pp. 137-151. In K. Maramorosch and R.E. Shope (eds.) Invertebrate Immunity. Academic Press, Inc., New York.

5. Anderson, R.S. 1977. Biochemistry and physiology of invertebrate macrophages in vitro, pp. 1-20. In L.A. Bulla and T.C. Cheng (eds.) Comparative Pathobiology, Vol. 3. Plenum Press, New York.

6. Salt, G. 1970. The Cellular Defense Reactions of Insects. Cambridge Univ. Press, London.

7. Ratner, S. and S.B. Vinson. 1983. Phagocytosis and encapsulation: cellular immune responses in Arthropoda. Amer. Zool. 23:185-194.

8. Soderhall, K. and V.J. Smith. 1986. Prophenoloxidase-activating cascade as a recognition and defense system in Arthropods, pp. 251-285. In A.P. Gupta (ed.) Hemocytic and Humoral Immunity in Arthropods. John Wiley and Sons, New York.

9. Johansson, M.W. and K. Soderhall. 1985. Exocytosis of the prophenoloxidase acivating system from crayfish haemocytes. J. Comp. Physiol. B. 156:175-181.

10. Chadwick, J.S. and G.B. Dunphy. 1986. Antibacterial and antiviral factors in arthropod hemolymph, pp. 287-330. In A.P. Gupta (ed.) Hemocytic and Humoral Immunity in Arthropods. John Wiley and Sons, New York.

11. Boman, H.G. and D. Hultmark. 1981. Cell-free immunity in insects. TIBS. 6:305-309.

12. Okada, M. and S. Natori. 1983. Purification and characterization of an antibacterial protein from haemolymph of Sarcophaga peregrina (flesh-fly) larvae. Biochem. J. 211:727-734.

13. Amirante, G.A. 1986. Agglutinins and lectins of Crustacea, pp. 359-380. In A.P. Gupta (ed.) Hemocytic and Humoral Immunity in Arthropods. John Wiley and Sons, New York.

14. McKay, D., C.R. Jenkin, and D. Rowley. 1969. Immunity in the invertebrates. I studies on the naturally occurring haemagglutinins in the fluid from invertebrates. Aust. J. Exp. Biol. Med. Sci. 47:125-134.

15. Hapner, K.D. and M.R. Stebbins. 1986. Biochemistry of arthropod agglutinins, pp. 227-250. In A.P. Gupta (ed.) Hemocytic and Humoral Immunity in Arthropods. John Wiley and Sons, New York.

16. Barondes, S.H. 1983. Soluble lectins: a new class of extracellular proteins. Science. 223:1260-1264.

17. Lis, H. and N. Sharon. 1986. Lectins as molecules and tools. Ann. Rev. Biochem. 55:35-67.

18. Jurenka, R., K. Manfredi, and K.D. Hapner. 1982. Haemagglutinin activity in Acrididae (grasshopper) haemolymph. J. Insect Physiol. 28:177-181.

19. Hapner, K.D. 1983. Haemagglutinin activity in the haemolymph of individual Acrididae (grasshopper) specimens. J. Insect Physiol. 29:101-106.

20. Hapner, K.D. and M.A. Jermyn. 1981. Haemagglutinin activity in the haemolymph of Teleogryllus commodus (Walker). Insect Biochem. 11:287-295.

21. Komano, H., D. Mizuno, and S. Natori. 1980. Purification of lectin induced in the hemolymph of Sarcophaga peregrina larvae on injury. J. Biol. Chem. 255:2919-2924.

22. Anderson, R.S., N.K.B. Day, and R.A. Good. 1972. specific hemagglutinin and a modulator of complement of cockroach hemolymph. Infection and Immunity. 5:55-59.

23. Scott, M.T. 1972. Partial characterization of the hemagglutinating activity in hemolymph of the american cockroach Periplaneta americana. J. Invert. Path. 19:66-71.

24. Amirante, G.A. 1976. Production of heteroagglutinins in haemocytes of Leucophaea maderae L. Experientia. 32:526-528.

25. Stynen, D., M. Peferoen, and A. DeLoof. 1982. Proteins with haemagglutinin activity in larvae of the colorado beetle Leptinotarsa decemlineata J. Insect Physiol. 28:465-470.

26. Lackie, A.M. 1981. The specificity of the serum agglutinins of Periplaneta americana and Schistocerca gregaria and its relationship to the insects immune response. J. Insect Physiol. 27:139-143.

27. Yeaton, R.L.W. 1980. Lectins of a north american silkmoth (Hyalophora cecropia): their molecular characterization and developmental biology. Ph.D. Diss., Univ. of PA.

28. Suzuki, T. and S. Natori. 1983. Identification of a protein having hemagglutinating activity in the hemolymph of the silkworm, Bombyx mori. J. Biochem. 93:583-590.

29. Pendland, J.C. and D.G. Boucias. 1986. Characteristics of a galactose-binding hemagglutinin (lectin) from hemolymph of Spodoptera exigua larvae. Dev. Comp. Immunol. 10:477-487.

30. Renwrantz, L. 1983. Involvement of agglutinins (lectins) in invertebrate defense reactions: the immuno-biological importance of carbohydrate-specific binding molecules. Dev. Comp. Immunol. 7:603-608.

31. Sharon, N. 1984. Carbohydrates as recognition determinants in phagocytosis and in lectin-mediated killing of target cells. Biol. Cell. 51:239-246.

32. Gupta, A.P. 1986. Arthropod immunocytes, pp.3-59. In Gupta (ed.) Hemocytic and Humoral Immunity in Arthropods. John Wiley and Sons, New York.

33. Rowley, A.F., N.A. Ratcliffe, C.M. Leonard, E.H. Richards, and L. Renwrantz. 1986. Humoral recognition factors in insects, with particular reference to to agglutinins and the prophenoloxidase system, pp. 381-406. In A.P. Gupta (ed.) Hemocytic and Humoral Immunity in Arthropods. John Wiley and Sons, New York.

34. van der Knaap, W.P.W., L.H. Boerrigter-Barendsen, D.S.P. van der Hoeven, and T. Sminia. 1981. Immunocytochemical demonstration of a humoral defense factor in blood cells (amoebocytes) of the pond snail Lymnaea stagnalis. Cell Tissue Res. 219:291-296.

35. Renwrantz L. and A. Stahmer. 1983. Opsonizing properties of an isolated hemolymph agglutinin and demonstration of lectin-like recognition molecules at the surface of hemocytes from Mytilus edulis. J. Comp. Physiol. 149:535-546.

36. Vasta, G.R., T.C. Cheng, and J.J.Marchalonis. 1984. A lectin on the hemocyte membrane of the oyster (Crassostrea virginica). Cellular Immunol. 88:475-488.

37. Amirante, G.A. and F.G. Mazzalai. 1978. Synthesis and localization of hemagglutinins in hemocytes of the cockroach Leucophaea maderae L. Dev. Comp. Immunol. 2:735-740.

38. Lackie, A.M. 1986. Biochemical characterization and biological role of a serum lectin from the cockroach Periplaneta americana. Dev. Comp. Immunol. 10:631 (Abstract only).

39. Komano, H., R. Nozawa, D. Mizuno, and S. Natori. 1983. Measurement of Sarcophaga peregrina lectin under various physiological conditions by radioimmunoassay. J. Biol. Chem. 258:2143-2147.

40. Sminia, T., W.P.W. van der Knaap, and P. Edelenbosch. 1979. the role of serum factors in phagocytosis of foreign particles by blood cells of the freshwater snail Lymnaea stagnalis. Dev. Comp. Immunol. 3:37-44.

41. Renwrantz, L. and W. Mohr. 1978. Opsonizing effect of serum and ablumin gland extracts on the elimiation of human erythrocytes from the circulation of Helix pomatia. J. Invert. Path. 31:164-170.

42. Renwrantz, L., W. Schancke, H. Harm, H. Erl, H. Liebsch, and J. Gercken. 1981. Discriminative ability and function of the immunobiological recognition system of the snail Helix pomatia. J. Comp. Physiol. 141:477-488.

43. Anderson, R.S., B. Holmes, and R. Good. 1973. In vitro bactericidal capacityof Blaberus craniifer hemocytes. J. Invert. Path. 22:127-135.

44. Scott, M.T. 1971. Recognition of foreignness in invertebrates II. In vitro studies of cockroach phagocytic haemocytes. Immunology. 21:817-828.

45. Rowley, A.F. and N.A. Ratcliffe. 1980. Insect erythrocyte agglutinins, *in vitro* opsonization experiments with *Clitumnus extradentatus* and *Periplaneta americana* haemocytes. Immunology. 40:483-492.

46. Stebbins, M.R. and K.D. Hapner. 1985. Preparation and properties of haemagglutinin from haemolymph of Acrididae (grasshoppers). Insect Biochem. 15:451-462.

47. Hapner, K.D. Unpublished results.

48. Kohler, G. and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 256:495-

49. Vaitukaitis, J.L. 1981. Production of antisera with small doses of immunogen: multiple intradermal injections. Methods Enzymol. 73:46-52.

50. Knudsen, K.A. 1985. Proteins transferred to nitrocellulose for use as immunogens. Anal. Biochem. 147:285-288.

51. Laemmli, U.K. and M. Favre. 1973. Maturation of the head of Bacteriophage T4. J. Mol. Biol. 80:575-599.

52. Gershoni, J.M. and G.E. Palade. 1983. Protein blotting: principles and applications. Anal. Biochem. 131:1-15.

53. Morrissey, J.H. 1981. Silver stain for proteins on polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. 117:307-310.

54. Willingham, M.C. and I. Pastan. 1985. *An Atlas of Immunofluorescence in Cultured Cells*. Academic Press, Inc., New York.

55. Markwell, M.K., S. Haas, N.E. Tolbert, and L.L. Bieber. 1981. Protein determination in membrane and lipoprotein samples: manual and automated procedures. Methods Enzymol. 72:296-303.

56. Gupta, A.P. 1979. *Insect Hemocytes*, A.P. Gupta (ed.) Cambridge Univ. Press, Cambridge.

57. Sternberger, L.A. 1986. *Immunocytochemistry*, Third ed. John Wiley and Sons, New York.

APPENDICES

APPENDIX A

Western blot horseradish peroxidase immunostain procedure:

1. Nitrocellulose containing electrophoresed, electro-transfered grasshopper hemagglutinin is incubated in Tris-tween buffer (0.05 M Tris, 0.5 M NaCl, 0.05% Tween-20, pH 7.2) for 2 hr, room temperature, or overnight at 4°C.

2. Incubate nitrocellulose in primary antibody on rocking table (i.e. monoclonal antibody concentrate that has been diluted 1:5 in Tris-tween) for 2 hr, 37°C, or overnight at room temperature

3. Wash nitrocellulose on rocking table, three times, 10 minutes each, with Tris-tween.

4. Incubate nitrocellulose in horseradish-conjugated secondary antibody (i.e. HRP-conjugated goat-anti mouse IgG, diluted 1:1000 in Tris-tween) for 2 hr, room temperature.

5. Wash nitrocellulose three times, 10 minutes each, in Tris-tween.

6. Wash nitrocellulose briefly in Tris buffer (no tween).

7. Incubate nitrocellulose in developer solution:
   - 25 ml Tris buffer
   - 5 ml of a 3 mg/ml 4-chloro-1-napthol in methanol solution
   - 12.5 ul 30% hydrogen peroxide (0.015%)

   Incubate 1 hr, room temperature.

8. Rinse nitrocellulose with distilled water and dry between paper towels.

APPENDIX B

Indirect immunofluorescence protocol for monolayer hemocytes:

1. Fix monolayer hemocytes in 3.7% formalin in DPBS for 1 hr, 4°C.

2. Wash monolayers three times, one minute each, to remove fixative.

3. Incubate monolayers in primary antibody on rocking table for 1 hr, room temperature. Primary antibody is the mouse monoclonal supernate, diluted from concentrate to original strength with DPBS containing 0.1 M galactose. Control monolayers are incubated in mouse whole serum (1 ug/ml) in complete IMDM containing 0.1 M galactose.

4. Wash three times, five minutes each, in DPBS containing 0.1 M galactose.

5. Incubate in secondary antibody for 1 hr, room temperature, with rocking. Secondary antibody is an FITC-conjugated goat-anti mouse IgG diluted 1:100 in complete IMDM containing 0.1 M galactose.

6. Wash three times, five minutes each, in DPBS containing 0.1 M galactose.

7. Mount under anti-fade glycerol-PBS (Citifluor Limited, London) and observe.