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Host Defense Mechanisms in the Crayfish: The Effect of Injection with Live or Killed
Bacteria

A thesis
presented to
the faculty of the Department of Health Sciences
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Master of Science in Biological Sciences

by
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May 2003

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Keywords: Invertebrate Immunology, Hemocytes, Phagocytosis, LPS and mannose
receptors

ABSTRACT

Host Defense Mechanisms in the Crayfish: the Effect of Injection with Live or Killed
Bacteria

By

Kimberly R. Goins

An increase in attachment of SRBCs to *Procambarus clarkii* hemocytes has been shown after the crayfish were injected with a live or killed *Pseudomonas* strain RS2b. The increase in attachment occurred at 8 hours post injection and peaked at 24 hours for both experimental groups. The population of hemocytes with receptors for LPS and mannose also increased at 8 hours post injection and peaked at 24 hours for both experimental groups. At 96 hours post injection the number of receptor bearing hemocytes and hemocytes bound to SRBCs began to decrease to the level of the control for both groups. The protein concentration of hemolymph from the experimental groups remained stable at 8 and 24 hours post injection and increased at 96 hours. The correlation of the protein concentration increase at 96 hours with the decrease of receptor bearing hemocytes may be due to the degranulation of the receptor bearing hemocytes.

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CHAPTER 1

INTRODUCTION

The immune system can be divided into two categories: the innate immune system and the adaptive immune system. The innate immune system which is also known as the natural immune system is the first line of defense against an infection. The innate immune system responds non-specifically to foreign invasion. This means that it is not able to distinguish one microbial species from another. Another characteristic of the innate immune response is that it does not have memory and, therefore, is not improved after repeated exposure to the same invading microbe. Some examples of the innate immune response include phagocytosis, inflammation, and clotting mechanisms (1, 2). The adaptive immune system, also known as the acquired immune system, is a specific immune response. Not only can it distinguish between two different microbial species but it can also distinguish between different serotypes within a species. The adaptive immune response does have memory and therefore is improved by repeated exposure to the same microbe. After a second exposure to the same microbe, the adaptive immune system will respond stronger and faster than it did after the first exposure. T-cells, B-cells, and antibodies are all part of the adaptive immune system (1, 2).

The adaptive immune system found in humans and other vertebrates is an antigen specific immune response. Lymphocytes such as T-cells and B-cells will respond specifically to invading foreign materials. These foreign materials can be living pathogens or simple non-living antigens. Each lymphocyte will carry receptors for one specific antigen. Therefore, the millions of lymphocytes in the body collectively carry millions of different receptor specificities because each lymphocyte carries only one antigen receptor specificity (1, 2).

Antibodies are produced by an important group of lymphocytes known as B-cells. With the help of T-cells, B-cells will produce antibodies when the B-cell binds with high affinity to the antigen for which it possesses the specific receptor. The antibodies will then bind to the antigen. When a B-cell binds to its specific antigen, it also becomes activated and will proliferate making many clones of itself. The B-cell will also

differentiate into plasma cells which secrete antibodies and memory cells (2). The memory cells are important during subsequent exposure to the same antigen. After the antigen concentration is reduced to a level that is not high enough to elicit an adaptive immune response, the level of antibody production gradually declines. However, memory B-cells will remain and will mount a response if the infection reoccurs. This second response will be stronger and faster than the first response (2).

T-cells are an important group of lymphocytes that respond to intracellular pathogens and help activate B-cells. Certain pathogens such as viruses, some intracellular bacteria, and parasites will replicate inside cells rather than in the blood and extracellular spaces where antibodies are active. T-cells are important in destroying these intracellular pathogens (2). T-cells will recognize and bind to a specific antigen that is presented on the surface of the infected cell. Cytotoxic T-cells will kill the infected cell after it binds to the presented antigen. T_H1 cells recognize macrophages that are infected by bacteria such as *Mycobacterium tuberculosis* which can live in the vesicles of the macrophage. The T_H1 cells stimulate the macrophage to destroy the bacteria by inducing fusion of the vesicle with a lysosome as well as induce other phagocytic mechanisms. T-helper cells are also important in B-cell activation. Before most B-cells will proliferate and differentiate, they require a signal from a T-helper cell that will recognize the same antigen that the B-cell recognizes (2).

Invertebrates in general and crayfish in particular have only nonspecific immune responses to infectious agents. Crayfish immune responses do not show specificity and, therefore, cannot distinguish one microbe from another. The immune response is the same for all invading microbes. Crayfish immune responses do not show long-term memory. For example, if a crayfish is exposed to the same microbe more than once, the immune response is not improved after each identical exposure. Also, the cells that are found in the adaptive immune response, such as T-cells and B-cells, are not present in crayfish (3).

Even though crayfish do not have long-term memory responses, short-term changes in the host-defense mechanisms can occur after exposure to bacteria. McKay and Jenkin have shown that phagocytic activity in crayfish can be increased for a short period of time after exposure to bacteria (4). In their assay they used the Australian

crayfish *Parachanna bicarinatus*. First, the crayfish were immunized with four doses of endotoxin. The crayfish hemolymph was extracted at 30 minutes, 60 minutes, and 90 minutes post injection and allowed to incubate with erythrocytes. Phagocytic activity of the erythrocytes by the hemocytes was shown to increase over the 90 minute time period. This assay showed that after immunization of the crayfish phagocytic activity increased over a short period of time as compared to non-immunized crayfish (4).

Crayfish have an elaborate innate immune system. Some of the important mechanisms of the innate immune system in crayfish include phagocytosis, antibacterial peptide production, the pro-phenyloxidase system, clotting mechanisms, and encapsulation responses (3). Phagocytosis is an important mechanism by which infectious agents are removed. First the microbe will attach to the surface of the phagocyte. The phagocyte will then engulf the microbe forming a vesicle inside the phagocyte known as a phagosome. Lysosomes inside of the phagocyte will then fuse with the phagosome. The lysosomes contain digestive enzymes which will digest the microbe in the phagolysosome (1, 2).

Antibacterial peptides are another important innate immune defense against infectious agents. Antibacterial peptides bind to bacteria and form pores in the cell membrane of the bacteria causing the cells to lyse. Some antibacterial peptides are found in the hemolymph of invertebrates permanently while others are produced after the invertebrate is exposed to bacteria or bacterial products (5). The antibacterial peptides of insects have been studied in the greatest detail. Insects have about 15 different antibacterial peptides that can be induced after the insects are exposed to bacteria or bacterial products. For example, cecropins are peptides that are produced in insects and have a broad spectrum of activity against both Gram positive and Gram negative bacteria (6, 7, 8). Little is known about antibacterial peptide production in crayfish, although they are assumed to be present (5).

The pro-phenyloxidase system is similar to the complement system in vertebrates. The pro-phenyloxidase system produces proteins that can adhere to the surface of foreign particles. This process of coating a particle with proteins is known as opsonization. The opsonized particle is then easier for a phagocyte to bind to and engulf. The pro-phenyloxidase system also produces lytic and degranulating factors. It is an enzyme

cascade that is released in the presence of LPS (lipopolysaccharide) or foreign polysaccharide and involves at least two serine proteases. In crayfish the pro-phenyloxidase system resides within semigranular and granular hemocytes in the secretory vesicles (9).

Crayfish and other invertebrates use clotting mechanisms as a non-cellular chemical method of sealing off an infection or a puncture wound (10). Sealing off an infection is important in preventing the spread of the infection. Sealing off a puncture wound is important to prevent leaking of the hemolymph. Encapsulation responses use cellular methods to seal off infections. In this case phagocytes are used to create a wall around the infection to prevent its spread (3, 9).

A population of hemocytes, about 5-8% of adherent cells, expresses receptors for both mannose and LPS. This population of cells contains granules that are believed to be released after exposure to either microbes or microbial products (11). Many active molecules are added to the hemolymph after these granules are released from the hemocytes. These active molecules are involved in the innate immune system of the crayfish (3, 9). The activation of this cell population, followed by degranulation, may be a key step in many effector mechanisms that are seen in the innate immune system of the crayfish.

The hemocytes of crayfish can be divided into three categories. The hyaline cells are the most prevalent type of hemocytes found in the hemolymph of crayfish. About 75-77% of hemocytes are hyaline cells. Hyaline cells are phagocytic cells that do not contain granules and have a hyaline cytoplasm. Hyaline means that the cell is transparent, colorless, and contains no granules. Hyaline cells also have a high nucleocytoplasmic ratio (3, 12). About 8-9% of hemocytes are known as semigranular cells. Semigranular cells contain small oval granules that are visible when stained with Wright's stain. These granules are part of the pro-phenyloxidase system of the innate immune response of the crayfish. Semigranular hemocytes have a low nucleocytoplasmic ratio. Semigranular cells are fusiform or ovoid in shape and have little phagocytic ability. These specific cells can degranulate when directly exposed to LPS and β -1,3-glucans. They are also involved in the encapsulation response to sealing off infections (12). Between 15-16% of hemocytes are granular hemocytes. Granular

hemocytes contain large granules and have a low nucleo-cytoplasmic ratio. These granules are larger than those of semigranular hemocytes and are also involved in the pro-phenyloxidase system. Granular hemocytes have no phagocytic activity and mainly have a circular shape. The granules can be released into the hemolymph when exposed to foreign protein or glycoprotein such as β -1,3-glucans (3, 12).

The objective of the work presented here is to examine the effects of injection of live or killed bacteria on the innate immune system of the crayfish *Procambarus clarkii*. The effect of injection of bacteria on the percentage of hemocytes with sheep red blood cells attached will be analyzed by performing attachment assays. The effect of injection of bacteria on antibacterial production will also be investigated. LPS and mannose receptor bearing hemocytes will be examined to see if an increase in this cell population occurs after injection with live or killed bacteria. The working hypothesis in our lab is that when hemocytes are exposed to bacteria or bacterial products the bacteria will attach to the hemocyte via the mannose and LPS receptors. The attachment will then cause the hemocyte to degranulate releasing granules which contain chemicals involved in the prophenyloxidase system and clotting mechanisms. The chemicals in the granules may also be involved in activating phagocytic hemocytes causing them to phagocytose the invading bacteria. This would result in an increase in phagocytic and attachment activity by the hemocytes after exposure to bacteria. The granules may also be responsible for antibacterial peptide production. The chemicals in the granules may act upon an unknown hemocyte population causing the hemocytes to produce antibacterial peptides. Another possibility is that the granules may contain antibacterial peptides which are released after degranulation. The antibacterial peptides will attack invading bacteria by attaching to the bacterial membrane and forming pores causing the bacteria to lyse. This hypothesis will be supported if an increase in the receptor bearing hemocyte population, antibacterial peptide production, and percentage of attachment to sheep red blood cells by hemocytes is observed and correlated.

CHAPTER 2

MATERIALS AND METHODS

Animals

For all of the experiments the red swamp crayfish, *Procambarus clarkii*, were used. The crayfish were purchased through Carolina Biological Supply Co. in Burlington, NC from Waubun Laboratories in Shriever, LA. The animals were kept in fresh water aerated aquariums at room temperature (25°C) and were fed twice weekly with guinea pig food pellets from The Hartz Mountain Corporation in Secaucus, NJ.

Preparation of Reagents

Each of the following reagents was made and mixed thoroughly in a beaker with a stir bar before being sterilized.

Crayfish Anticoagulant Buffer (13)

For a 100ml solution 0.82g NaCl, 1.80g glucose, 0.88g trisodium citrate, 0.50g citric acid and 0.37g EDTA were mixed together in a large beaker containing 70ml double distilled H₂O using a stir bar. The solution was then brought to 100ml using double distilled H₂O. The pH was adjusted to 4.60. The solution was then filter sterilized with a filter pore size of 0.2 um and stored at 4°C.

Crayfish Saline (14)

For a 500ml solution 5.84g NaCl, 201.4mg KCl, 555mg CaCl₂, 264.4mg MgCl₂+6 H₂O and 84.0mg NaHCO₃ were mixed together in 450ml double distilled H₂O in a large beaker using a stir bar until completely dissolved. The total volume was adjusted to 500ml using double distilled H₂O. The pH of the solution was adjusted to 6.75. The crayfish saline was then autoclaved for sterilization and stored at 4°C.

TMN-FH insect media

For a 250ml solution 12.8g of powder cell culture medium was dissolved in 200ml double distilled H₂O in a beaker using a stir bar until completely dissolved. The solution was then adjusted to 250ml using double distilled H₂O. The media was then filter sterilized with a filter pore size of 0.2 um and stored at 4°C. The final concentration of the media was 51.2g/L.

Streptomycin Dilutions

For a 2ml solution with a concentration of 13.7 mg/ml, 27.4mg of streptomycin was dissolved in 2ml of distilled H₂O. The solution was vortexed to ensure homogeneity. Dilutions were made by dissolving 1ml of the original solution in 9ml distilled H₂O for dilution 1 and 1ml of dilution 1 into 9ml of distilled H₂O to make dilution 2. The dilutions were thoroughly mixed before each transfer. The two dilutions were used in the antibacterial peptide assay.

Nutrient Agar

For a 100ml solution 2.3g of nutrient agar is dissolved in 100ml distilled H₂O in a 250ml volumetric flask. The solution is then autoclaved with a stir bar for sterilization. After autoclaving the nutrient agar solution is allowed to cool in a 45°C water bath.

Bovine Albumin Standard Protein

For a concentration of 1mg/ml, 2 mg of bovine albumin was dissolved in 2 ml distilled H₂O and mixed thoroughly. The original solution was used to make dilutions of standard protein.

Lipopolysaccharide

One mg of lyophilized, chromatically purified FITC-LPS (lipopolysaccharide) was obtained from Sigma Chemical Co., St. Louis, MO and was stored at 4°C. The FITC-LPS was prepared from the smooth LPS of *E.coli*, serotype 0111:B4. It contained 2-10µg FITC per mg of LPS. The FITC-LPS was reconstituted and diluted in 0.15M NaCl to the concentration of 5 µg ml⁻¹ LPS (1g LPS in 200ml NaCl). The LPS powder

had been detoxified, lyophilized, and chromatographically purified. Dilutions were then stored at -80°C.

Mannose

Five mg of FITC-mannose was obtained from Sigma Chemical Co., St. Luis, MO and was stored at 4°C. The FITC-mannose was reconstituted and diluted to a concentration of 1µg ml⁻¹. Dilutions were then stored at -80°C.

Wright's Stain Solution

To stain the hemocytes Wright's stain was thoroughly mixed with Giordano's buffer in a ratio of 2:1.

Glycine Saline Buffer (Immunofluorescence Mounting Medium)

Fourteen grams of glycine, 0.7g NaOH, 17g NaCl, and 1g sodium azide were dissolved in 500 ml double distilled H₂O with a stir bar (pH 8.6). Thirty ml of the buffer was mixed with 70 ml 30% glycerol.

Bovine Albumin Standard Protein

One mg of bovine albumin was dissolved in 1 ml of distilled water to obtain a concentration of 1 mg/ml standard protein.

Preparation of Bacterial Cultures

Pseudomonas strain RS2b

A *Pseudomonas* strain RS2b was isolated from the feces of the red swamp crayfish, *Procambarus clarkii* by Dr. Eric Mustain and stored at -80°C. The frozen *Pseudomonas* was briefly thawed and a sterile needle used to transfer the bacteria into nutrient broth. The *Pseudomonas* was incubated for 24 hours at 37°C in a shaker to aerate. After incubation the culture was streaked onto a plate to obtain isolated colonies and incubated for 24 hours at 37°C for 24 hours. The isolated colony was Gram stained

to verify purity and then inoculated onto a nutrient agar slant or into a nutrient broth and incubated for 24 hours at 37°C. The pure *Pseudomonas* was used in the experiments.

Staphylococcus aureus

S. aureus was inoculated into sterile nutrient broth and incubated at 37°C for 24 hours in a shaker for aeration. The *S. aureus* was then used in the experiments.

Micrococcus luteus

M. luteus was inoculated into sterile nutrient broth and incubated at 30°C or 37°C for 24 hours. The *M. luteus* was then used in the experiments. *M. luteus* grew best when inoculated from a slant.

Bleeding of Crayfish and Preparation of Hemocytes

All crayfish were anesthetized in an ice bath for several minutes before being bled. Only healthy adult crayfish were used for bleeding and immunization. Immunized crayfish were kept in separate labeled aquaria during the course of the experiments. Crayfish immunized with live bacteria were kept separate from crayfish immunized with killed bacteria. Control crayfish were separated from immunized crayfish. Colored nail polish was used to mark crayfish that had been bled so that they would not be reused for a later time point. To withdraw the hemolymph from the crayfish the abdominal hemocoel is punctured using a 22 gauge sterile needle. A 3cc sterile syringe (Fisher Scientific, Pittsburgh, PA) containing 0.5ml of crayfish anticoagulant buffer is used to collect the hemolymph of 2 crayfish from each of the experimental groups (live and killed) and the control. The hemolymph from the individual syringes was then deposited into relevant sterile disposable centrifuge tubes (Fisher Scientific, Pittsburgh, PA) labeled live, killed, or control. The tubes were then centrifuged in a swinging bucket centrifuge at 1,200 rpm for 10 minutes at 4°C. After centrifugation the supernatant containing hemolymph and anticoagulant buffer was removed with a Pasteur pipette and stored at 0°C. The pellet containing hemocytes was resuspended in 2 ml TMN-FH insect media. A 0.100 mm deep hemocytometer (Hausser Scientific) was used to count the cells from each of the groups using a 400x objective lens of an Olympus BH-2-RHCA microscope (Olympus

Optical Co., LDT, Japan). If needed, the cells were diluted to 5×10^6 cells/ml with TMN-FH insect media and mixed with a Pasteur pipette. The cell suspension was then overlaid onto sterile 22 x 22 mm coverslips in the bottom of an ethanol treated 6 well tissue culture plate (Fisher Scientific, Pittsburgh, PA). Each well was labeled live, killed, or control and the relevant hemocyte suspensions were used for each of the three coverslips. The cells were incubated to allow adherence with the coverslips at room temperature (25°C) for one hour for the attachment assay and 20°C for 30 minutes for the receptor assay. After incubation the coverslips were washed five times in three changes of crayfish saline to removed non-adherent cells.

Attachment Assay

The washed coverslips were then placed into clean labeled wells of the 6 well plate and flooded with a 5% sheep RBC suspension. The coverslips were allowed to incubate with the SRBCs for 1 hour at room temperature to allow attachment of the SRBCs to the hemocytes. The coverslips were again rinsed 5 times in 3 changes of crayfish saline to remove unattached SRBCs. The coverslips are then fixed in 100% methanol for 2 minutes at 25°C. After fixation, the coverslips were allowed to completely air dry standing on labeled paper towels. Dried coverslips were then stained with Wright's stain for 30 minutes in staining jars. The coverslips were washed 5 times in 3 changes of distilled H₂O to remove excess stain and allowed to completely dry as before. The dried coverslips were placed in staining jars with xylenes to clear for 5 minutes and then mounted onto labeled slides using permount. Before viewing, the slides are allowed to dry for 24 hours. Several fields are viewed on each slide with a 400x objective lens. Total hemocytes in each field are counted and recorded. Hemocytes with RBCs attached are also counted and recorded. The attachment assay was performed identically for each time point.

Receptor Assay

Washed coverslips are fixed in 100% methanol for 2 minutes and washed again 10 times in 3 changes of crayfish saline. The fixed hemocytes were then incubated in an ethanol treated 6 well plate with FITC-LPS for 30 minutes in the dark at room

temperature. FITC-LPS functioned as the ligand for possible LPS receptors on the hemocytes. For the mannose receptor assay, the fixed hemocytes were incubated with FITC-mannose (1 $\mu\text{g/ml}$) for a ligand for mannose receptors on the hemocytes for 30 minutes at room temperature in the dark. After incubation, the coverslips (for both LPS and mannose receptor assay) were rinsed 10 times in 3 changes on crayfish saline to remove unbound ligand. The coverslips were mounted face down onto glass slides using immunofluorescence mounting medium. For each slide 250 hemocytes (about 5 fields) were counted and recorded as before. Using fluorescence microscopy with 400x magnification, the number of fluorescing hemocytes in each field were counted and recorded.

Antibacterial Peptide Assay

Two flasks (100ml each) of nutrient agar were made, autoclaved, and allowed to cool in a 45°C water bath for 30-45 minutes. Bacterial cultures of *S. aureus*, *Pseudomonas* and/or *M. luteus* are stained and counted using a hemocytometer. The bacterial cultures (either *S. aureus* and *Pseudomonas* or *M. luteus* and *Pseudomonas*) are mixed with the nutrient agar (one culture/agar) using a stir bar so that the concentration is 10^4 cells/ml. The nutrient agar with the bacteria is poured into labeled disposable Petri dishes and allowed to solidify. Three plates for each bacteria (6 plates total) are made. Using a core borer wells are punched into the agar (5-6 wells/plate) and labeled. Dilutions of streptomycin are made by dissolving 27.4 mg streptomycin in 2 ml distilled H₂O and vortexing to mix completely. One ml of the streptomycin is added to 9 ml distilled H₂O and mixed thoroughly for dilution 1. One ml of dilution 1 is added to 9 ml of distilled water and mixed thoroughly for dilution 2. Streptomycin dilution 1 and 2 are used as the positive control and are added to two of the wells. Anticoagulant buffer is used as a negative control since it is present in the hemolymph samples. The anticoagulant buffer is diluted by mixing 1 ml buffer with 1 ml distilled H₂O. The diluted buffer is added to one well. The remaining wells are used for hemolymph samples (live, killed, and control at 8 hours, 24 hours, and 96 hours post-injection). For all the samples the volume added to the wells was 35 μL . Below is an example of a plate used in the antibacterial peptide assay:

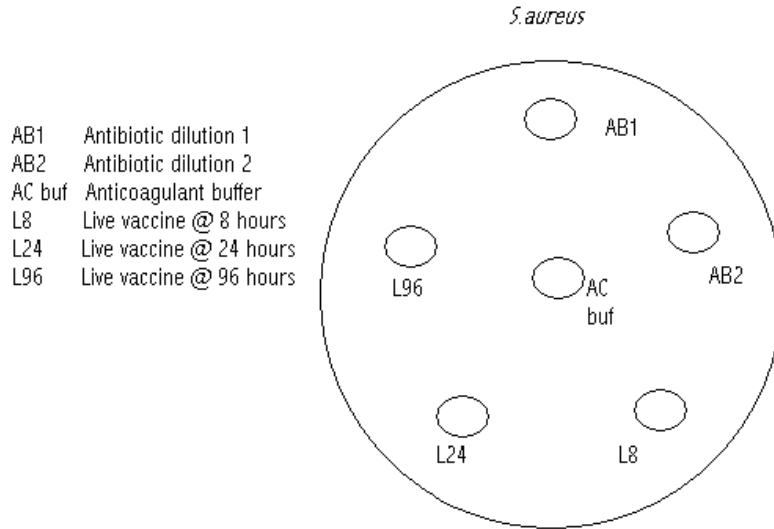


Figure 1. Example of Antibacterial Peptide Assay Plate.

The plates were incubated at 37°C for 48 hours. After incubation the plates were observed for zones of inhibition.

Immunization Protocol

Pseudomonas that was isolated from the feces of the crayfish was used to immunize the crayfish. After incubation, the cells were centrifuged at 1200 rpms for 20 minutes at 10°C. The cell pellet was resuspended in 1 ml crayfish saline and counted using a hemocytometer. The cell suspension was then diluted to a concentration of one million cells per ml. The cell suspension was separated into 2 aliquots. One aliquot was autoclaved and used as the killed bacterial vaccine. The other aliquot was left untreated and used as the live vaccine. Crayfish saline was used as a control. The crayfish were then injected with the vaccines. Six crayfish were injected with 1 ml of the killed vaccine, 6 crayfish were injected with 1 ml of the live vaccine, and 6 crayfish were injected with 1 ml of crayfish saline control. The immunized crayfish were used in all 3 assays. To decide which concentration of vaccine to use mortality studies were performed. Concentrations of 10^6 , 10^7 , and 10^8 bacterial cells/ ml were injected into the

crayfish. Because the crayfish were able to tolerate the 10^6 concentration it was used for the immunization protocol. The mortality studies were repeated twice.

Response of Crayfish to Live Bacteria

Pseudomonas was streaked on a nutrient agar plate to obtain isolated colonies. The isolated colonies were Gram stained to check for purity and then grown in a nutrient broth at 37°C for 24 hours. Cells were counted and diluted to concentrations of 10^8 , 10^7 , and 10^6 bacterial cells/ml. Two crayfish were injected with the 10^8 concentration, 2 crayfish were injected with the 10^7 concentration, and 2 crayfish were injected with the 10^6 concentration. Each injection was 1 ml each. As a control 2 crayfish received an injection of crayfish saline. The crayfish were then observed for mortality.

Protein Concentration Assay

A solution of the standard protein bovine albumin was made with a concentration of 1 mg/ml. Clean test tubes are labeled 1- 10. Into the test tubes 10-100 μg of the standard protein is added in increments of 10 (ex: 10 μg , 20 μg , ect.). The total volume of the standards is made to be 100 μl with distilled water. Another tube is used for the blank and only contains 100 μl distilled water. Nine more tubes are used for the unknown hemolymph samples from each of the experimental groups at each time point. Five microliters from each of the hemolymph samples are put into the corresponding labeled tubes. The total volume of the tubes is increased to 100 μl with distilled water. To all of the tubes 2 ml of the BCA (bicinchoninic acid) reagent is added and the tubes are mixed well and incubated at 37°C for 30 minutes. After incubation the absorbance of the blank, standards and hemolymph samples is measured at 562 nm wavelength. The blank absorbance measurement is subtracted from the absorbance measurements of the standards and hemolymph samples. A graph is made of the standard protein (standard absorbance Vs standard concentration) and used to find the concentration of the unknown hemolymph samples.

CHAPTER 3

RESULTS

Mortality Studies

To determine which concentration of bacteria to use in the immunization assay, mortality studies were performed. Using a pure culture of *Pseudomonas* dilutions were made to obtain concentrations of 10^6 , 10^7 , and 10^8 bacterial cells/ml. Two crayfish were injected with the 10^6 concentration, 2 crayfish were injected with the 10^7 concentration, and 2 crayfish were injected with the 10^8 concentration. As a control 2 crayfish were injected with a crayfish saline control. The mortality study was performed twice. In the first study 2 crayfish injected with the 10^8 concentration and 1 crayfish injected with the 10^7 concentration died within 2 weeks after exposure. In the second study 1 crayfish injected with the 10^8 concentration and 1 crayfish injected with the 10^7 concentration died within 2 weeks after exposure. Crayfish injected with the 10^6 concentration were able to tolerate the exposure without dying (Table 1). None of the control animals died. The 10^6 concentration was used in all of the immunization assays.

Table 1. Mortality Studies. Mortality studies demonstrated that crayfish were able to tolerate injections of 10^6 bacterial cells/ml without dying. None of the controls died.

| Concentration Bacterial cells/ml | Mortality Study 1 (% mortality) | Mortality Study 2 (% mortality) |
|-------------------------------------|------------------------------------|------------------------------------|
| 10^6 | 0% | 0% |
| 10^7 | 16.7% | 16.7% |
| 10^8 | 33.3% | 16.7% |

Effect of Injection of Live or Killed Bacteria on Attachment of Hemocytes to SRBCs

The attachment assay was performed to determine the effects of injection with live or killed bacteria on the percentage of hemocytes attached to sheep RBCs. The percentage of hemocytes with SRBCs attached was calculated from the data collected at each time point. The hemocytes were harvested at 8 hours post injection, 24 hours post

injection, and 96 hours post injection. The attachment assay was performed identically for each time point.

An increase in attachment of *P. clarkii* hemocytes to SRBCs was seen in the crayfish immunized with live or killed bacteria. At 8 hours post injection the percentage of hemocytes from the killed group attached to SRBCs was 7.1%. For the killed group at 8 hours post injection the percentage was 6.5%. At 24 hours post injection the attachment rate of the hemocytes exposed to live or killed bacteria peaked (20.2% and 19.8% respectively) and then began to return to the level of the control at 96 hours post injection (12.2% for the live group and 15.3% for the killed group) (Figures 1, 2, and 3). Hemocytes from control crayfish injected with crayfish saline did not show an increase in attachment to SRBCs at any of the time points. At 8 hours post injection the percentage of hemocytes with SRBCs attached was 3.4% for the control group. At 24 hours post injection 5.4% of the control hemocytes were attached to SRBCs. At 96 hours post injection 4.1% of the control group hemocytes had SRBCs attached. Hemocytes exposed to live bacteria exhibited a slightly higher percentage of attachment than those exposed to the killed bacteria (Figures 1 and 2).

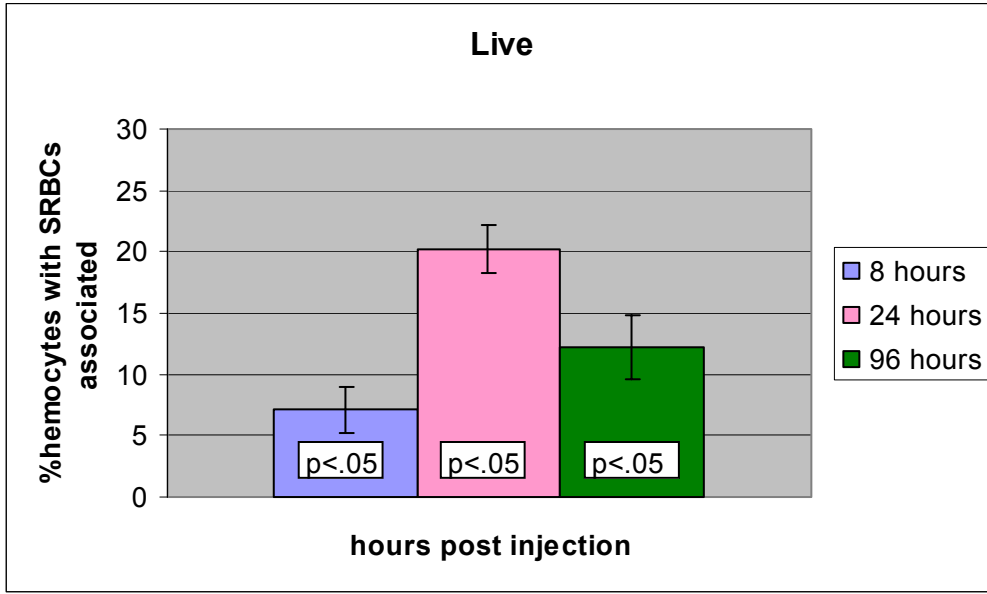


Figure 2. Effect of Injection of Crayfish with Live Bacteria on Percentage of Hemocytes with SRBCs Attached at 8, 24, and 96 Hours Post Injection. Combination of 6 experiments. Significant difference ($p<.05$) shown by comparing each time point to control data using t-test.

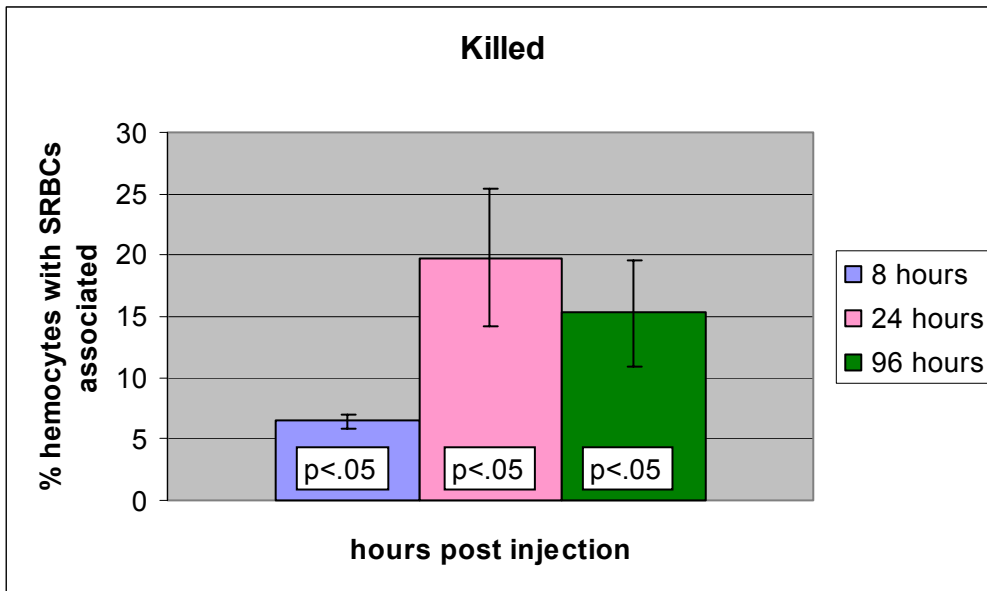


Figure 3. Effect of Injection of Crayfish with Killed Bacteria on Percentage of Hemocytes with SRBCs Attached at 8, 24, and 96 Hours Post Injection. Combination of 6 experiments. Significant difference ($p<.05$) shown by comparing each time point to control data using t-test.

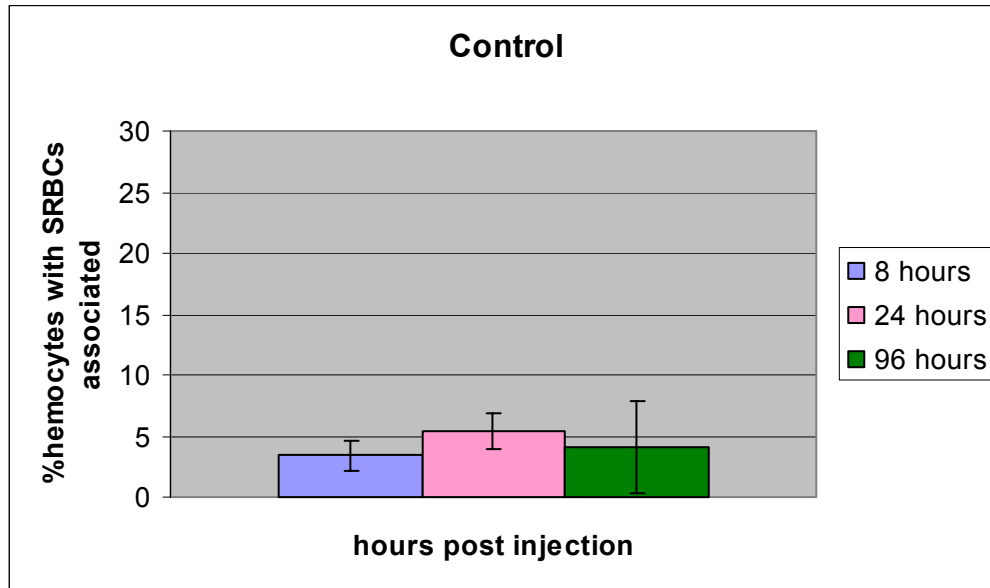


Figure 4. Effect of Injection of Crayfish with Crayfish Saline Control on Percentage of Hemocytes with SRBCs Attached at 8, 24, and 96 Hours Post Injection. Combination of 6 experiments.

Effect of Injection of Live or Killed Bacteria on Production of Antibacterial Peptides

The antibacterial assay was performed to determine the effect of injection with live or killed bacteria on the production of antibacterial peptides by the crayfish hemocytes. The pour plate method was used to make nutrient agar plates containing 10^4 bacterial cells per ml. Six 3mm wells were punched into the agar and labeled. Two of the wells contained dilutions of streptomycin. One well contained a dilution of crayfish anticoagulant buffer (1 part buffer to 1 part distilled water). The other three wells were used for the hemolymph samples from each of the experimental groups and the control group. The plates were then incubated at 37°C for 48 hours.

An attempt to develop an antibacterial peptide assay for the crayfish hemocytes was unsuccessful. The results of the crayfish antibacterial peptide assay were not reproducible. Some reduced bacterial colony size was seen around the wells containing the crayfish hemolymph but these results were not consistent. No clear zones of inhibition were seen. The protocol for insect antibacterial peptides did not work for the crayfish hemocytes.

Efforts were made to modify the antibacterial assay. Different volumes of hemolymph were added to the wells. Well sizes were adjusted to conform to the new volumes. Hemolymph volumes ranged from 20 μ L to 100 μ L. The volume changes did not affect the results of the assay. Hemolymph including hemocytes was also used in the assay rather than using the hemolymph alone to determine if the hemocytes had any antibacterial activity. The addition of hemocytes did not affect the results of the assay. To determine if different bacteria might be susceptible to the hemolymph samples *M. luteus* was used in the assay. The *M. luteus* was inhibited by the hemolymph samples and the positive controls. However, it was also inhibited by the negative control and the control group hemolymph samples.

The hemolymph samples were also used to determine the protein content of the hemolymph from the 2 experimental groups. A standard protein solution was made from bovine albumin. The hemolymph samples were made identically by using 5 μ L of hemolymph. BCA reagent is added to the tubes and incubated. The BCA reagent contains copper (Cu^{2+}) which is reduced to Cu^+ creating a purple color when protein is present. The more protein that is present the more copper is reduced producing the purple color. The absorbance of the samples and standards is then measured (Table 2). A higher absorbance reading is associated with an increase in copper reduction. Therefore, a higher absorbance reading will mean that more protein is present. The graph of the standard protein is used to find the concentration of protein in the hemolymph samples from each group at each time point.

An increase in the protein content of the hemolymph samples was seen in the crayfish injected with live and killed bacteria (Figures 5, 6, and Table 3). The increase in protein content for the two groups was seen at 96 hours post injection. The protein concentration of the group injected with live bacteria increased from 87.28 and 89.46 micrograms/ml at 8 and 24 hours respectively to 137.72 micrograms/ml at 96 hours post injection. The protein concentration of the group injected with killed bacteria increased from 83.15 and 97.72 micrograms/ml at 8 and 24 hours respectively to 153.59 micrograms/ml at 96 hours post injection. At 8 and 24 hours post injection the protein content was similar to that of the control group. The control group did not show a significant increase in protein concentration at any time point (Figure 7 and Table 3).

Table 2. Estimation of Protein in Hemolymph Samples. Absorbance measurements for the standard protein samples and the hemolymph unknown samples. Standard sample measurements were adjusted for the blank.

| Standards | | Hemolymph samples | |
|-----------|------------|-------------------|------------|
| sample | absorbance | sample | absorbance |
| blank | 0.140 | L8 | 0.400 |
| 1 | 0.048 | L24 | 0.410 |
| 2 | 0.095 | L96 | 0.632 |
| 3 | 0.141 | K8 | 0.381 |
| 4 | 0.172 | K24 | 0.448 |
| 5 | 0.222 | K96 | 0.705 |
| 6 | 0.259 | C8 | 0.472 |
| 7 | 0.321 | C24 | 0.374 |
| 8 | 0.348 | C96 | 0.480 |
| 9 | 0.440 | | |
| 10 | 0.447 | | |

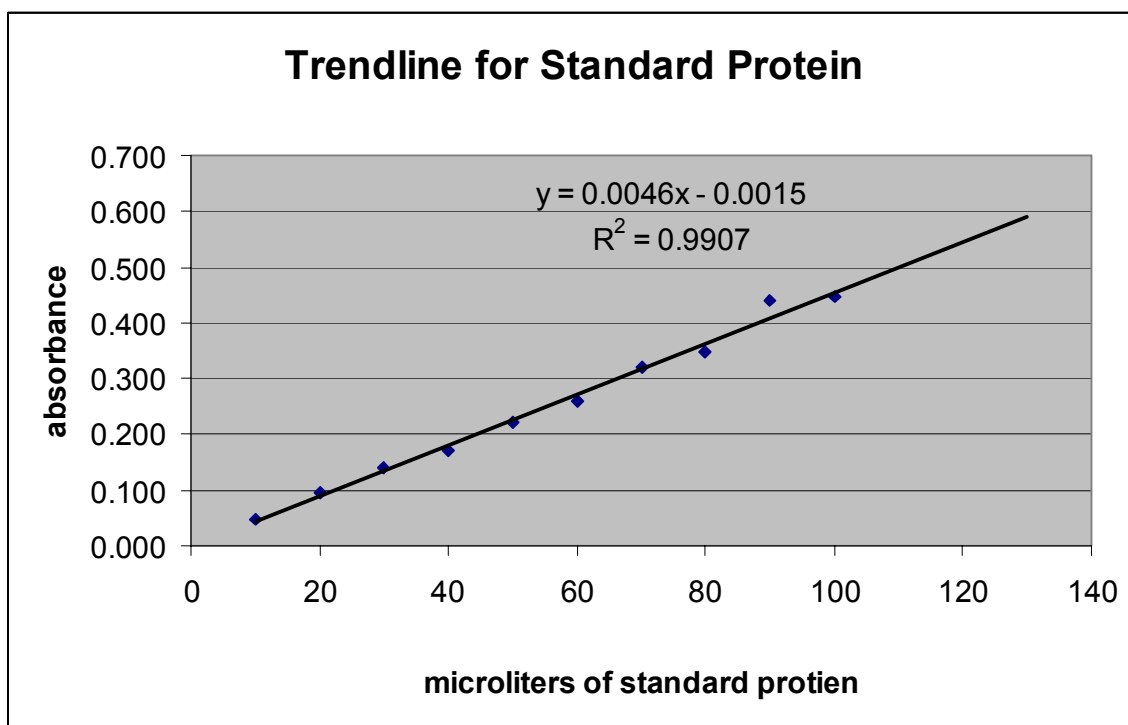


Figure 5. Trendline for the Standard Protein Bovine Albumin. The standard protein graph was used to find the concentration of protein in the hemolymph samples.

Table 3. Protein Concentration of Hemolymph Samples. Protein concentrations for the hemolymph samples were found using the equation from the graph of the standard protein. An increase in protein concentration occurred in the hemolymph samples as the time points increased for both experimental groups.

| hemolymph samples | protein concentration (μL) |
|-------------------|---|
| L8 | 87.28 |
| L24 | 89.46 |
| L96 | 137.72 |
| K8 | 83.15 |
| K24 | 97.72 |
| K96 | 153.59 |
| C8 | 102.93 |
| C24 | 81.63 |
| C96 | 104.67 |

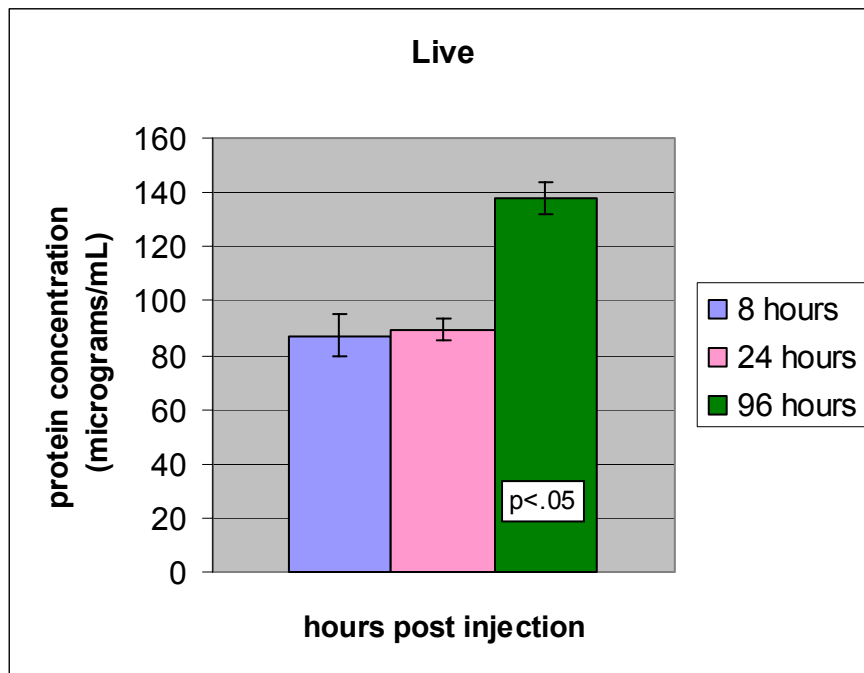


Figure 6. Protein Concentration of the Hemolymph Samples from the ‘Live’ Experimental Group at 8, 24, and 96 Hours Post Injection. Significant difference ($p < .05$) shown by comparing each time point to control data using t-test.

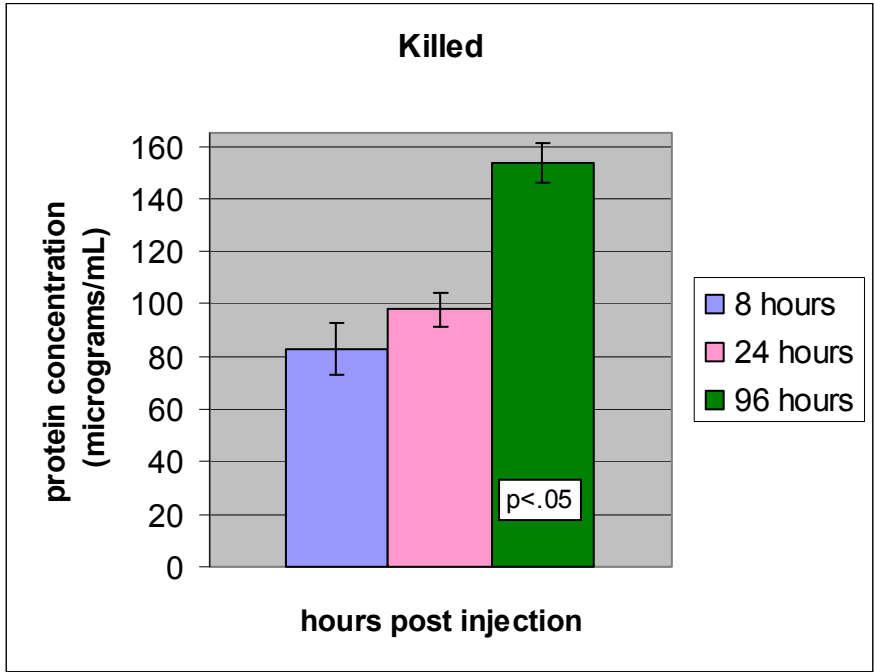


Figure 7. Protein Concentration of the Hemolymph Samples from the ‘Killed’ Experimental Group at 8, 24, and 96 Hours Post Injection. Significant difference ($p<.05$) shown by comparing each time point to control data using t-test.

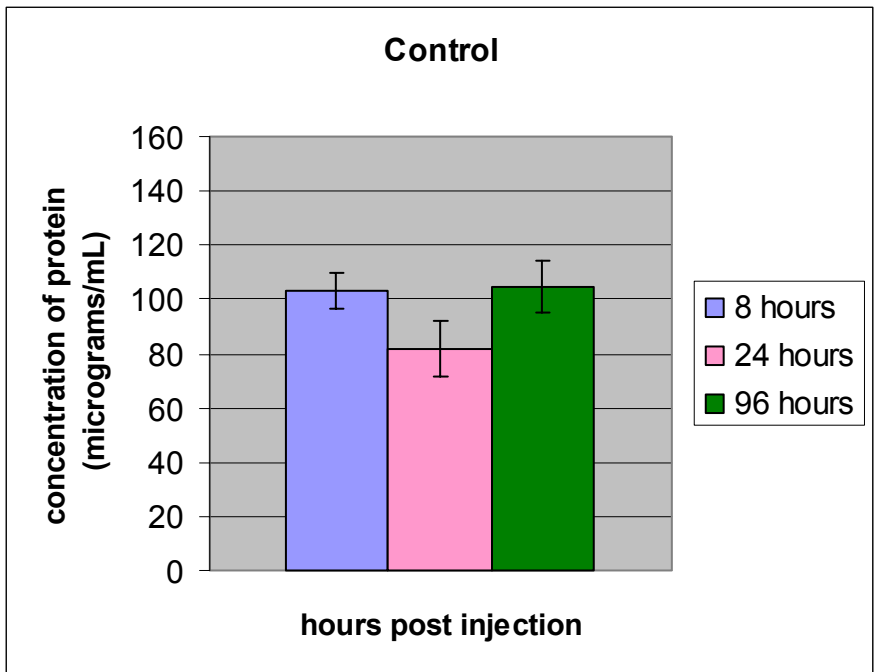


Figure 8. Protein Concentration of the Hemolymph Samples from the ‘Control’ Group at 8, 24, and 96 Hours Post Injection.

Effect of Injection of Bacteria on Hemocytes with Receptors for LPS or Mannose

Between 5 and 8 % of hemocytes that adhere to the glass coverslips express receptors for both mannose and LPS (11). The receptor assay was performed to determine if exposure to live or killed bacteria would increase the number of hemocytes that express receptors for both LPS and mannose. For this assay fluorescing LPS and fluorescing mannose were used. When the LPS or mannose attaches to the hemocyte, the hemocyte will fluoresce and can be viewed with a microscope using the 400x objective lens. The number of fluorescing hemocytes is counted for each of the experimental groups and the control group.

An increase in the population of cells that express the receptors for both LPS and mannose was seen in both the group injected with live bacteria and the group injected with killed bacteria at 8 and 24 hours post injection. The group injected with live bacteria had 12.8% fluorescent cells at 8 hours and 24.0% at 24 hours post injection for the mannose assay. For the LPS assay the live group had 14.3% fluorescent cells at 8 hours and 26.8% at 24 hours post injection. The group injected with the killed bacteria showed a slightly greater increase in the LPS receptor cell population (Fig. 9, 12) compared to the group injected with the live bacteria (Fig. 8, 11). The group injected with the killed bacteria had 14.1% fluorescent cells at 8 hours and 28.4% at 24 hours post injection for the mannose assay. For the LPS assay the killed group had 15.0% fluorescent cells at 8 hours and 29.0% at 24 hours post injection. The greatest increase of the receptor bearing cell population occurred at 24 hours post-injection (Fig. 8, 9, 11, 12). At 96 hours post-injection the receptor bearing cell populations from both groups began to return to the baseline of 5-8% (Fig. 8, 9, 11, 12). The control group injected with crayfish saline did not show a significant increase across the time points (Fig. 10, 13). The control group receptor cell population remained at 5-8% for each time point.

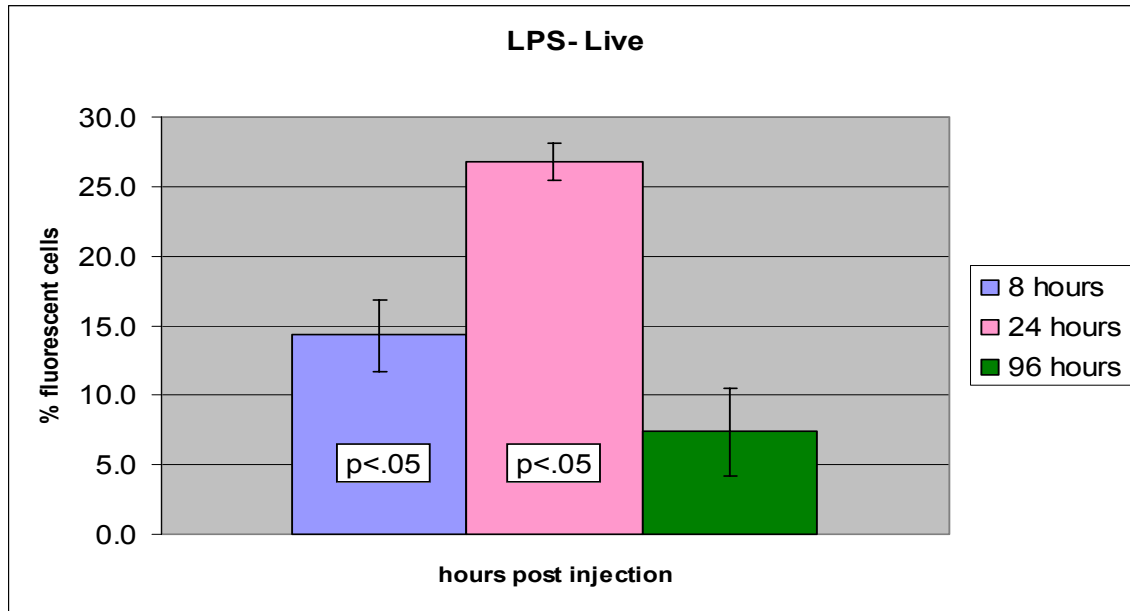


Figure 9. Effect of Exposing Hemocytes from the ‘Live’ Experimental Group to Fluorescing LPS at 8, 24, and 96 Hours Post Injection. Combination of 3 experiments. Significant difference ($p < .05$) shown by comparing each time point to control data using t-test.

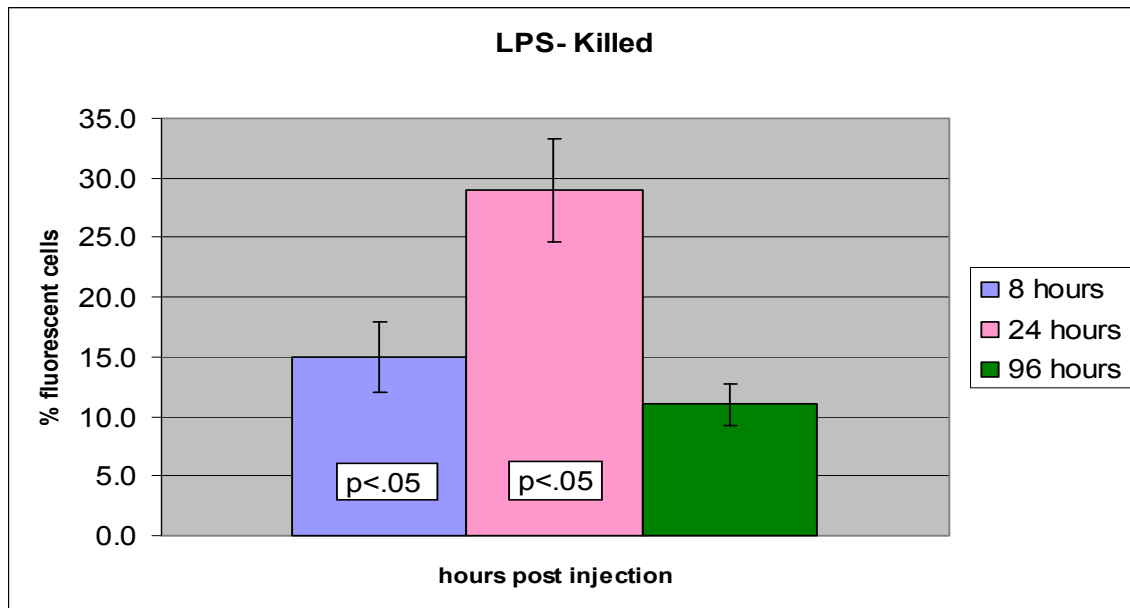


Figure 10. Effect of Exposing Hemocytes from the ‘Killed’ Experimental Group to Fluorescing LPS at 8, 24, and 96 Hours Post Injection. Combination of 3 experiments. Significant difference ($p < .05$) shown by comparing each time point to control data using t-test.

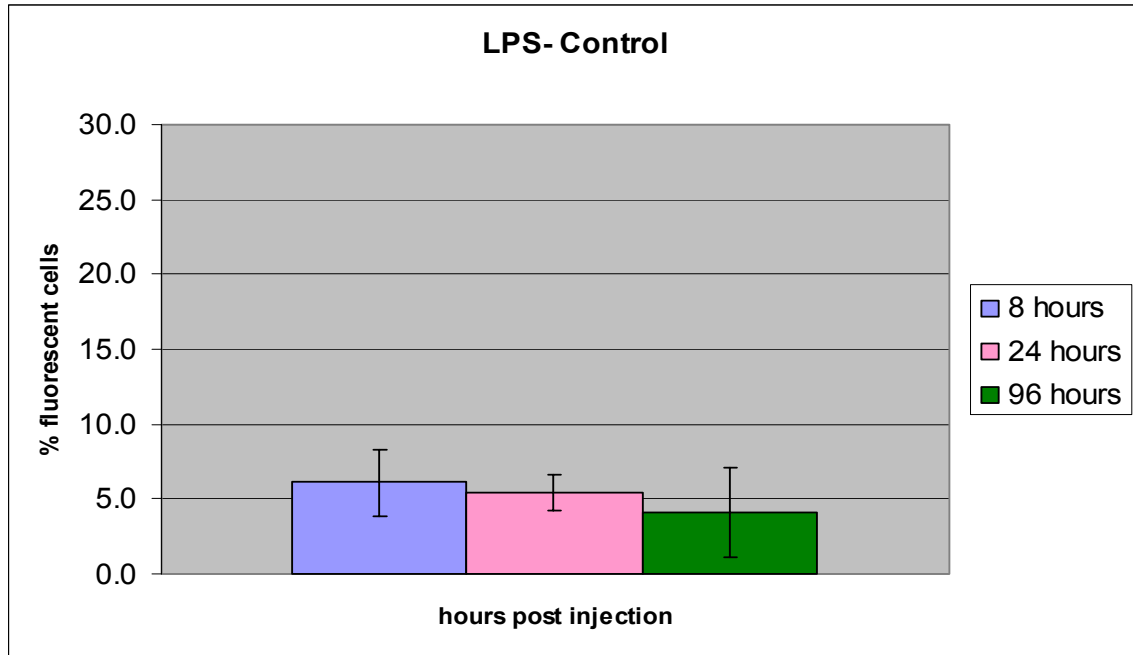


Figure 11. Effect of Exposing Hemocytes from the 'Control' Group to Fluorescing LPS at 8, 24, and 96 Hours Post Injection. Combination of 3 experiments.

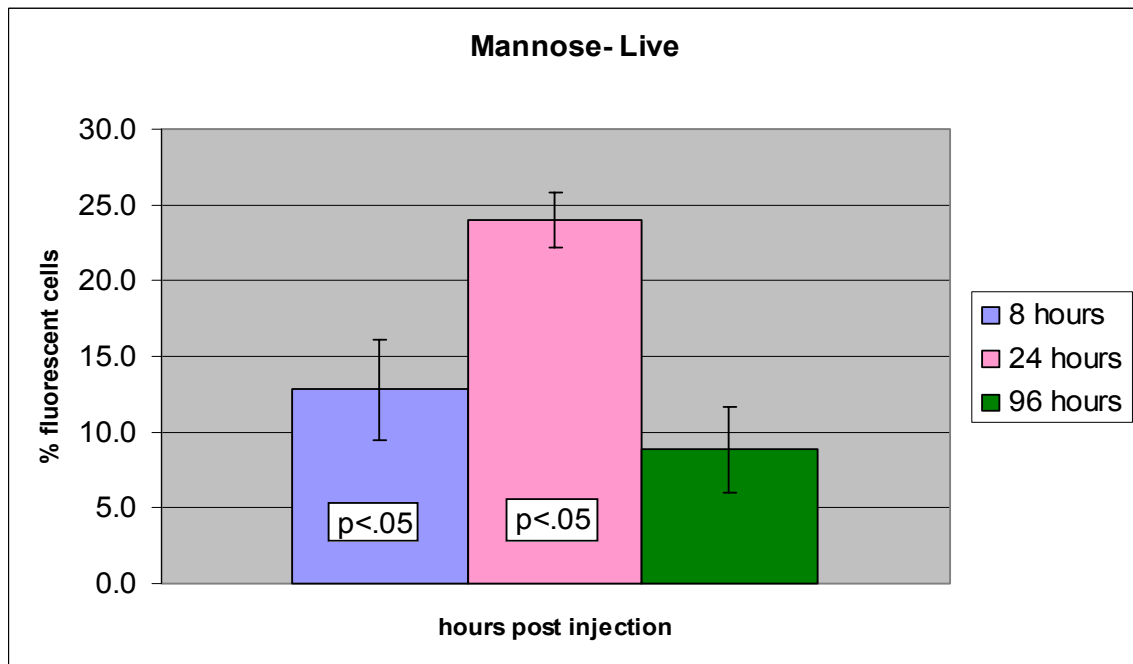


Figure 12. Effect of Exposing Hemocytes from the 'Live' Experimental Group to Fluorescing Mannose at 8, 24, and 96 Hours Post Injection. Combination of 3 experiments. Significant difference ($p < .05$) shown by comparing each time point to control data using t-test.

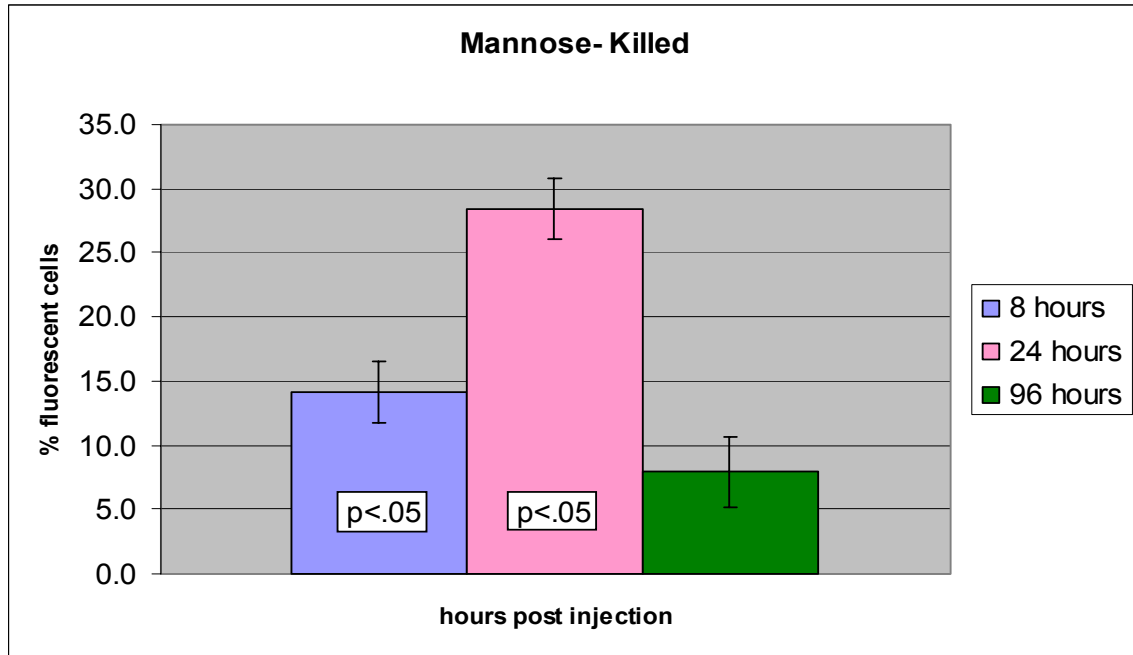


Figure 13. Effect of Exposing Hemocytes from the ‘Killed’ Experimental Group to Fluorescing Mannose at 8, 24, and 96 Hours Post Injection. Combination of 3 experiments. Significant difference ($p<.05$) shown by comparing each time point to control data using t-test.

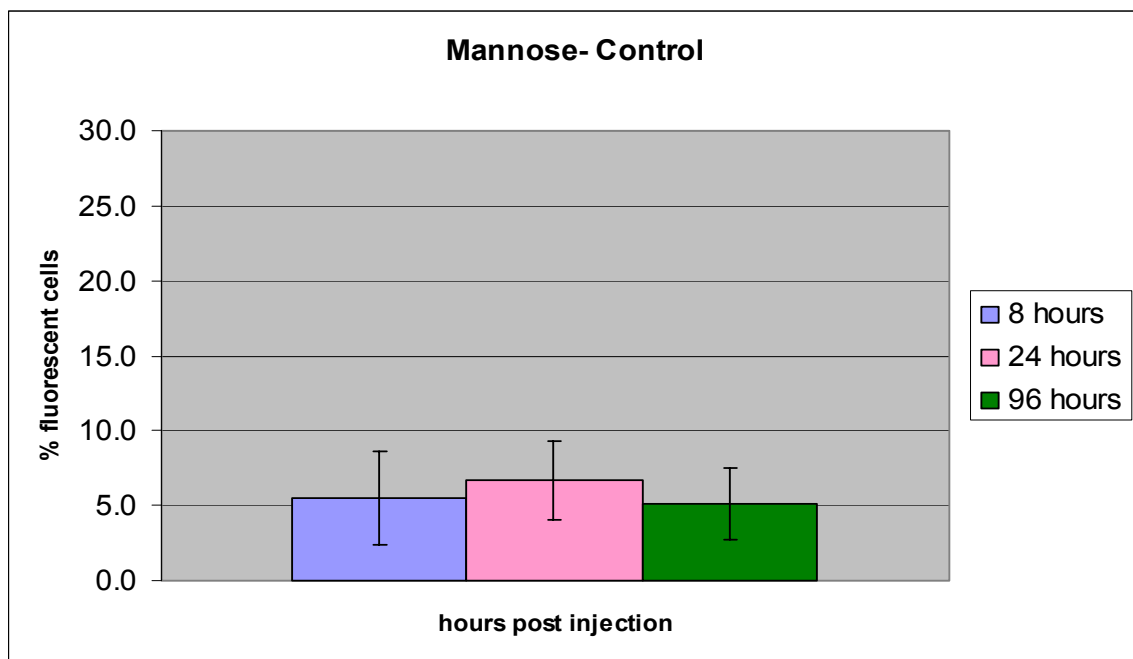


Figure 14. Effect of Exposing Hemocytes from the ‘Control’ Group to Fluorescing Mannose at 8, 24, and 96 Hours Post Injection. Combination of 3 experiments.

Correlation of Receptor Assay with Protein Concentration Assay

The receptor assay for both LPS and mannose was compared with the protein concentration assay (Figures 14 and 15). As the cell population with the LPS and mannose receptors increased at 8 and 24 hours post injection the protein concentration of the hemolymph remained stable. When the receptor cell population decreased in number at 96 hours post injection returning to the level of the control the protein concentration of the hemolymph increased.

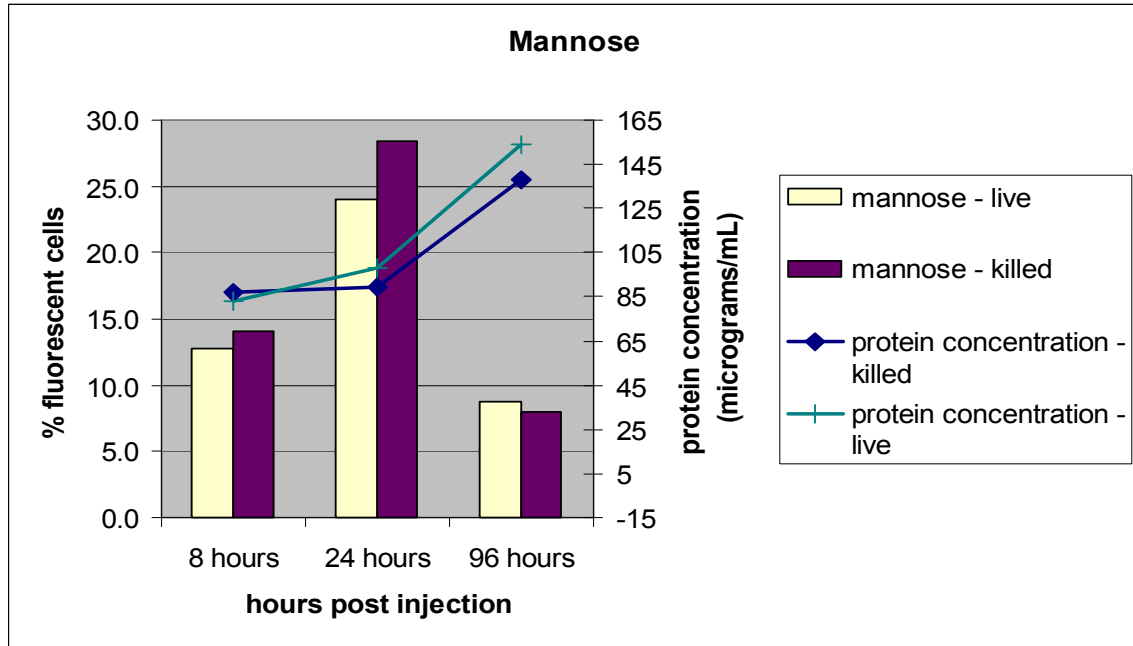


Figure 15. Comparing the Mannose Receptor Assay with the Protein Concentration Assay. As the population of cells expressing the receptor for mannose decrease, the protein concentration increases at 96 hours post injection.

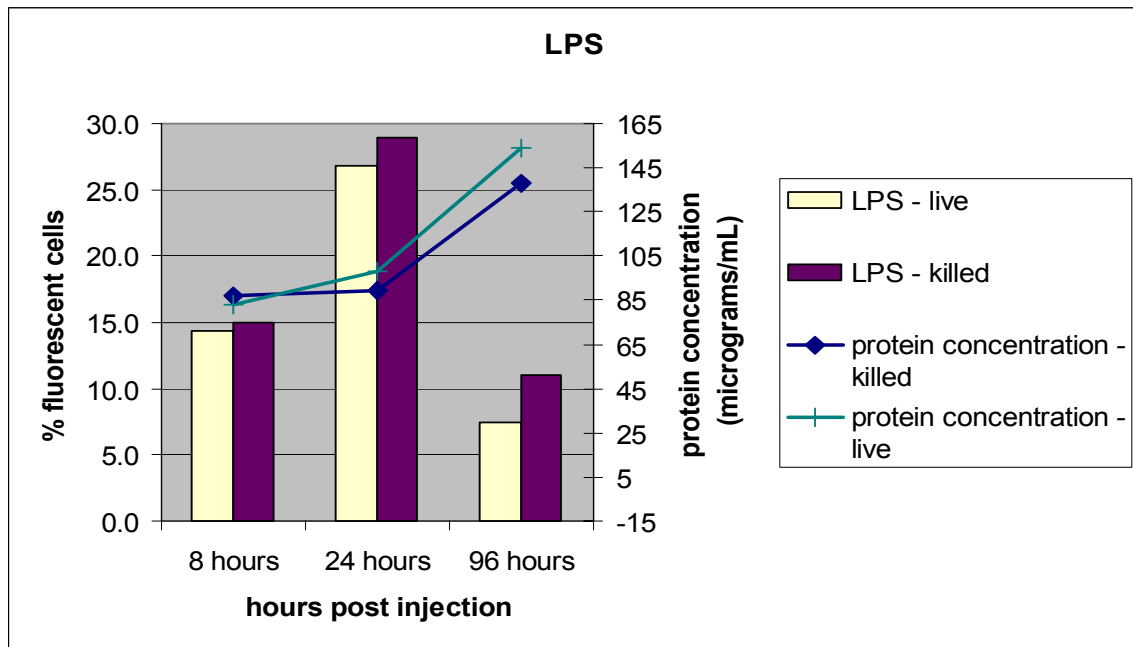


Figure 16. Comparing the LPS Receptor Assay with the Protein Concentration Assay. As the population of cells expressing the receptor for LPS decrease, the protein concentration increases at 96 hours post injection.

CHAPTER 4

DISCUSSION

Though crayfish and other invertebrates only display a primitive innate immune system, they are able to protect themselves from foreign invasion by bacteria, parasites, viruses, and other microbes. They are able to protect themselves by making short-term rather than long-term changes to their host defense mechanisms (3). Innate immune functions such as phagocytosis, antibacterial peptide production, the pro-phenyloxidase system, clotting mechanisms, and encapsulation responses are important short-term changes that the crayfish uses to defend itself from foreign attack (3, 4, 8). It is the goal of this research to better understand how some parts of the innate immune system work together to guard against invasion by foreign bacteria.

The hypothesis for this research is that after being exposed to bacteria or bacterial products some hemocytes will degranulate releasing granules which contain chemicals into the environment. First the bacteria or bacterial product will attach to the hemocyte via the LPS and/or mannose receptor causing degranulation of the hemocyte. The chemicals within the granules will then stimulate phagocytic hemocytes to become active and phagocytise the invading bacteria. This will cause an increase in phagocytic and attachment rates. The granules will also be important in antibacterial peptide production. Either the chemicals within the granules will stimulate an unknown population of hemocytes to produce the antibacterial peptides or the granules themselves will contain the antibacterial peptides. These peptides will attack invading bacteria by attaching to the cell membrane forming a pore and consequently lysing the bacteria. The increases in attachment of sheep RBCs to hemocytes, antibacterial peptide production, and the population of cells that produce the receptors for LPS and mannose after the crayfish are exposed to live or killed bacteria will suggest that this pathway may exist.

For the attachment assay an increase in the number of hemocytes with sheep RBCs attached was observed at 8 and 24 hours post injection with either live or killed bacteria (Figures 1 and 2). An increase was not seen in the control crayfish hemocytes injected with crayfish saline. The peak of phagocytic activity occurred at about 24 hours post injection. These results suggest that after exposure to bacteria or bacterial products

the phagocytic activity of hemocytes increases up to 24 hours after the exposure and then returns to the level of the control at 96 hours post injection.

The population of cells that express the receptors for both LPS and mannose also exhibited an increase in number after exposure to both live and killed bacteria at 8 hours and 24 hours post injection (Figures 6, 7, 9, and 10). The group injected with the crayfish saline control did not show an increase in the population of cells with the LPS and mannose receptors (Figures 8 and 11). The control group had between 4-8 % fluorescent cells which was consistent with results from previous experiments. For the groups injected with live or killed bacteria the increase in the cell population with the receptors peaked at 24 hours post injection then began to return to the level of the control at 96 hours post injection.

The increase in the population of cells bearing the receptors for LPS and mannose is consistent with the increase in phagocytic activity. Both the receptor cell population and the phagocytic activity increased at 8 and 24 hours post injection and both peaked at 24 hours post injection. These results support the hypothesis that activation of the population of cells with the LPS and mannose receptors due to exposure to bacteria followed by degranulation is a key step in activating phagocytic hemocytes to attach to and engulf the invading bacteria.

The decrease in the population of cells that have the LPS and mannose receptors at 96 hours post injection correlated with an increase in protein concentration (Figures 12 and 13). As the receptor cell population increased the protein concentration of the hemolymph remained stable. When the receptor cell population decreased in number at 96 hours post injection the protein concentration of the hemolymph increased. This increase in protein concentration may be a result of the degranulation of the receptor cell population. As the hemocytes bearing the receptors for LPS and mannose degranulate releasing the granules into the hemolymph the protein concentration in the hemolymph has increased.

Antibacterial peptides of insects have been studied in the greatest detail. The peptides are of low molecular weight, cationic, and have a broad spectrum of activity against Gram negative and/or Gram positive bacteria. These antibacterial peptides are induced after the organism is exposed to bacteria or bacterial products (5, 6). The

antibacterial peptides are stable to freezing but are unstable when exposed to heat. They are found in the granular and semigranular immune cells (5). The antibacterial peptide assay for insect antibacterial peptides was used to look for antibacterial peptides in crayfish. However, the results of this assay were not reproducible in the crayfish. Some reduced colony size was seen around the wells containing the hemolymph samples but clear zones of inhibition were not seen.

Future studies should be performed to investigate the accuracy of the working hypothesis. To improve the antibacterial peptide assay the hemolymph samples can be concentrated so that the antibacterial peptides will be in greater quantity and therefore may have a stronger reaction with the bacteria. Also, filter disks saturated with the hemolymph samples may be placed onto a lawn of bacteria on an agar plate, incubated and observed for zones of inhibition. Assay plates may be incubated at a lower temperature such as room temperature to ascertain if the antibacterial peptides are functional at 25°C.

Future studies can be performed to examine the effect of injection of live or killed bacteria on the population of cells that bear receptors for mannose and LPS. For our studies we looked at an increase in the population of receptor bearing cells. An increase in the number of receptors on individual hemocytes after exposure to live or killed bacteria should also be investigated. Hemocytes from crayfish exposed to live or killed bacteria could be compared to hemocytes from crayfish injected with a crayfish saline control to quantify the number of receptors for LPS and mannose on the individual hemocytes from each group.

An assay should be performed to look for direct evidence of degranulation of the hemocytes when the cells are stimulated with LPS and or mannose. For this assay the hemocytes would be incubated with either LPS or mannose. The cells would then be centrifuged at 10°C for 10 minutes as before. The supernatant would be used to look for the released products of degranulation. A substrate and optical density would be used to determine if a specific product of degranulation is present in the supernatant. The results would be presented as percent degranulation (15).

Time points may be altered to obtain a better picture of the response of the hemocytes to live and killed bacteria. An additional time point between 8 and 24 hours

post injection such as 12 or 16 hours post injection could be observed in both the attachment assay and the receptor assay. An additional time point between 24 and 96 hours post injection such as 48 hours post injection may also be observed for the assays. The additional time points may assist in a better understanding of when the reactions of hemocytes to bacteria are at their peak. For example the extra time points may show that the peak of attachment of hemocytes to sheep RBCs occurs not at 24 hours post injection but at 12 or 48 hours post injection.

It is important to study the components of the innate immune system to better understand the way in which crayfish and other invertebrates are able to protect themselves from bacteria, viruses, fungi, and parasites. It is also important to be able to study the innate immune system in the absence of the adaptive immune system to better understand how the innate immune system functions unaided. Better understanding of the innate immune system will assist in understanding how the adaptive immune system evolved from the more primitive innate immune system.

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