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 THE CELLULAR DEFENSE RESPONSES OF
RHODNIUS PROLIXUS AND TRIATOMA INFESTANS
 TO TRYPANOSOMA CRUZI
 presented by

Kathryn Bearden Smith

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THE CELLULAR DEFENSE RESPONSES OF RHODNIUS PROLIXUS
AND TRIATOMA INFESTANS TO TRYPANOSOMA CRUZI

By

Kathryn Bearden Smith

A THESIS

Submitted to
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ABSTRACT

THE CELLULAR DEFENSE RESPONSES OF RHODNIUS PROLIXUS AND TRIATOMA INFESTANS TO TRYPANOSOMA CRUZI

By

Kathryn B. Smith

Chagas' disease, American trypanosomiasis, is a significant health problem in humans and animals in Latin America. Trypanosoma cruzi, the etiologic agent, is transmitted by various Triatominae (Hemiptera; Triatomidae), including Triatoma infestans (Klug) and Rhodnius prolixus Stal. This study examined the defense responses of these insects in order to contribute information for the eventual improvement of vector control.

Differences between T. cruzi-infected and non-infected specimens were not observed in scanning and transmission electron microscope studies on the encapsulation response, light and electron microscope studies on the individual hemocytes, or polyacrylamide gels of hemolymph proteins. A nylon thread introduced into an insect's hemocoel was encapsulated by an inner melanized layer, followed by necrotic hemocytes, and an outer cellular or electron-dense layer. Surface morphology of these encapsulated threads showed hemocyte attachment and aggregation with an amorphous component. Plasmotocytes were the most abundant hemocytes, followed by granular hemocytes, prohemocytes, and oenocytoids.

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CHAPTER 1

INSECT IMMUNITY: ITS ROLE IN CHAGAS' DISEASE

INTRODUCTION

Chagas' disease, a widespread insect-associated disease in the Americas, is one of the most important causes of myocarditis in the world (Harwood and James, 1979). In 1982 there were an estimated 20 million human cases with some 65 million others at risk (WHO, 1982). The natural vectors of the parasite are several members of the subfamily Triatominae (Hemiptera: Reduviidae). Rhodnius prolixus Stal and Triatoma infestans (Klug) are two of the principal vectors (Zeledon and Rabinovich, 1981). Trypanosoma cruzi (Zoomastigophora: Trypanosomatidae), a flagellated parasite, is the etiologic agent (Chagas, 1909). Although Chagas' disease occurs most commonly in humans and animals in Latin America, cases are occasionally reported in the United States (Woody and Woody, 1955; Walton et al., 1956; Sullivan et al., 1969). Presently, there is no vaccine nor is chemotherapeutic protection available. Therefore, the only practical approach now available to reduce the risk of disease demands prevention, meaning vector control. Diagnostic techniques available include immunofluorescence, complement fixation, and xenodiagnosis.

Previously, extensive immunological studies of the vertebrate infection were made in hopes that these findings would lead to the development of a vaccine (Tarrant et al.,

1965; Dzbenski, 1974; Cunningham et al., 1978; Lima and Kierszenbaum, 1982; Schofield, 1982). Only within the past several decades has it been accepted that insects also elicit an immune response to pathogens. More sensitive serological tests allowed for this finding (Chadwick, 1967).

It was thought that observable hemolymph differences between infected and non-infected insects might lead to developments that alter the insects' immune system. This might eventually lead to an alternative means of control. Since so little is known regarding this vector-parasite relationship, any investigation of their interactions would be a contribution to the present knowledge.

The objective of this research was to determine the role of Trypanosoma cruzi in the cellular immune responses in two of its insect vectors, Rhodnius prolixus and Triatoma infestans. Hemocyte populations and encapsulation responses were examined in infected and control insects. Light microscopy was utilized to examine hemocyte populations, scanning electron microscopy to study surface detail of individual hemocytes and encapsulation responses, and transmission electron microscopy to observe the ultrastructure of capsule formation. Also, hemolymph proteins from infected and non-infected insects were compared by denaturing polyacrylamide gel electrophoresis to determine whether the protein constituency changed during infection.

LITERATURE REVIEW

Insect Immunity

Although the insect immune system is believed to lack the specificity of antigen-antibody reactions and to have no immunologic memory, it does have the ability to discern and eliminate foreign materials through cellular, if not humoral, immunity (Nappi, 1975). In vertebrates, antibodies circulate in the bloodstream and bind to specific antigens; lymphocytes and macrophages also react with foreign bodies. Vertebrate blood can often be used as a measure of illness and disease, whether the problem is vascular or not, whereas insect cellular reactions seem to protect only the hemocoel from infection. This is likely due to the open circulatory system in insects (Salt, 1970). However primitive an insect's immune system may seem compared to that of a vertebrate, the unquestionable success of the order Insecta suggests that their defense responses are effective.

The hemolymph of insects circulates throughout the body cavity, bathing the organs, and consists of a fluid plasma containing the hemocytes. Hemocyte functions include phagocytosis, encapsulation, melanization, wound healing, and possibly intermediate metabolism and storage (Salt, 1970; Chapman, 1982). In order to distinguish different hemocytes in the Triatominae, a classification scheme had to be devised. Many hemocyte types have been described, but much of the literature is controversial due to varying nomenclature systems and the different conditions and

techniques used in their descriptions. The most appropriate classification for R. prolixus and T. infestans is based on the general insect classification scheme of Jones (1962). He identified the best-defined hemocytes as the prohemocyte, plasmatocyte, and granulocyte. Other hemocyte types were also identified by Jones; the only one of these present in the Triatominae is the oenocytoid. Wigglesworth (1979), on the other hand, separated hemocytes of insects into 2 groups, the phagocytic types and the nonphagocytic types which he called oenocytoids.

In general, the Hemiptera have low numbers of hemocytes. Descriptions of the types of hemocytes are quite variable, due to the different methods of observation used by investigators. The following hemocyte descriptions are representative for the Triatominae: Prohemocytes are small round cells with a large nucleus (Gupta, 1979), and polymorphic cells which tend to form filopodia and vacuolate in vitro are called plasmatocytes (Wigglesworth, 1956; Yeager, 1945). Granulocytes have many distinct, round granules, a small, often obscured nucleus, and may lyse in vitro (Jones, 1962). However, Ravindranath (1977) stated that plasmatocytes and granular hemocytes are altered forms of each other. The two are often confused, and both can form vacuoles, phagocytize, undergo ameboid movements, and have bactericidal potentials. Oenocytoids, only observed occasionally, are large cells with a thick, homogeneous cytoplasm which often contains needlelike inclusions (Rizki

and Rizki, 1959).

The hemocytes of Rhodnius prolixus have been studied extensively by Wigglesworth (1933, 1955, 1956, 1973) and Jones (1965, 1967a, 1967b). Fourth instar R. prolixus has a total hemocyte count per cubic millimeter (THC) which varies from 800-2000 (Jones, 1962). The THC increases prior to ecdysis, decreases during it, then increases again after molting ends. Two to three days after feeding the hemocyte count decreases, and then increases four to five days later (Jones and Liu, 1961). According to Wigglesworth (1959), the hemocytes of R. prolixus do not readily coagulate. The encapsulation reaction in this species is less marked than in many other species of insects, probably due to the paucity of cells (Salt, 1963).

Melanin, an important component of the encapsulation reaction, is deposited around foreign material in the insect's hemocoel. A type of granulocyte, often called a coagulocyte, plays a key role in the activation of prophenoloxidase (tyrosinase), an enzyme in the melanization pathway (Gregoire, 1973). Hormonal imbalance during infection and hemocyte secretions have both been found to activate this enzyme (Nappi, 1973b; Pye, 1974), suggesting a humoral response. The humoral aspects of insect immunity are not well understood, although some insects are known to produce antimicrobial or lytic substances following certain types of infections (Nappi, 1975). The advent of amino acid analysis and the improvement of chromatography techniques

have helped to increase our understanding of this aspect of insect immunity, although there are still many unanswered questions.

The Encapsulation Response

An important aspect of the insect immune system is encapsulation. Insect hemocytes can either phagocytize or encapsulate foreign objects, depending on the size of the objects. It is therefore possible to study an insect's immune response by following either encapsulation or phagocytosis.

The encapsulation response, the easier reaction to study, involves several processes including aggregation, adhesion, and flattening of the hemocytes around a foreign object, and is often accompanied by melanin formation (Nappi, 1975). Upon contact with the foreign material, or xenograft, autolysis of the hemocytes occurs and the cytoplasm contents cover the xenograft (Poinar et al., 1968). The following regions have been found to comprise a capsule which surrounds the foreign material: An inner noncellular layer containing melanin, surrounded by necrotic hemocytes, flattened hemocytes, and an outer layer of loosely attached cells (Poinar et al., 1968).

Encapsulation may have originated as a means of preventing parasitism. Inert objects, such as nylon fibers, wood, and parasites, are encapsulated by insects (Salt, 1970; Wigglesworth, 1956). The stimulus initiating the encapsulation response seems to involve contact between the

hemocyte and the foreign object (Salt, 1970). Since the hemocytes of many insects do not react to eggs and larvae of their naturally occurring hymenopterous parasites, the parasites may exhibit a protective mechanism. Salt (1970) provided that a chemical is produced by these parasites which prevents the initial attachment of the hemocytes. The continual aggregation of hemocytes past the first cellular layer covering the foreign object can be explained by the change in cell surface properties (Grimstone et al., 1967), but what stimulates capsule termination has not yet been explained by cellular studies. The encapsulation reaction also plays a role in bodily maintenance since an insect's own damaged tissues are encapsulated (Salt, 1970).

A study of encapsulation in Triatoma infestans demonstrated that xenograft rejection was inhibited in those insects infected with Trypanosoma cruzi (Bitkowska et al., 1982). In fifth instar and adult insects with a 15-day infection of the Sonya strain of T. cruzi, implanted wire evoked a stronger reaction in the control insects than in the infected ones. These investigators concluded that the parasite inhibits the cellular defense reaction of its insect host, and suggested that some parasites may avoid destruction and develop within the insect's hemocoel following suppression of the immune system.

All types of hemocytes except oenocytoids are capable of phagocytizing foreign material (Jones, 1962). Phagocytosis, although more difficult to study than

encapsulation, could also provide useful information in regards to the insect immune response. Quantitative in vivo studies of phagocytosis, although informative, are not easily done in most insects, due to low total hemocyte counts and the tendency of hemocytes to localize near tissues, lowering the number of hemocytes in circulation (Jones, 1962). Brehelin and Hoffman (1980) did successfully study phagocytosis in Galleria mellonella and Locusta migratoria, both of which have high hemocyte counts. In vitro studies of phagocytosis, under strictly controlled conditions, could also provide more information on the immune response. Ratcliffe and Rowley (1974, 1975) performed in vitro studies on Galleria mellonella which were informative.

The research described herein was performed to better understand the encapsulation process in Rhodnius prolixus and Triatoma infestans.

CHAPTER 2

CHAGAS' DISEASE: THE PATHOGEN AND THE VECTOR

INTRODUCTION

Chagas' disease exists primarily as a zoonosis, but with the proximity of man to many wild and domestic animals, some of the vectors, such as Rhodnius prolixus and Triatoma infestans, became synanthropic, and as a result the incidence of human disease increased. The disease is endemic predominantly in rural areas where people live in close contact with animal carriers. The Triatominae are obligate blood feeders throughout their lives, requiring at least one blood meal at each instar. The native territory of Triatoma infestans extends from Lima, Peru, to 38 °S in Argentina, and as far west as Pernambuco, Brazil. Except for Panama and parts of Costa Rica, Rhodnius prolixus can be found from Mexico to northern South America (Zeledon, 1974).

A kissing bug feeding on an infected person or animal may ingest trypomastigotes with the bloodmeal. These ingested trypomastigotes transform into sphaeromastigotes and epimastigotes in the stomach of the insect (Brack, 1968). The epimastigotes multiply in the intestine, and in the rectum some of the epimastigotes differentiate into infective metacyclic trypomastigotes. While the kissing bug feeds, the feces are deposited onto the host and may be rubbed into the site of the bite, another opening in the skin, or the conjunctiva, whereupon the trypomastigotes then

enter the lymphatics, penetrate host tissues, and develop into amastigotes, the vertebrate intracellular forms. The amastigotes multiply intracellularly, eventually causing the cells to rupture, and are released as trypomastigotes. These may be ingested by another kissing bug, starting the cycle once again.

There exist conflicting reports regarding the presence or absence of intracellular hemocytic stages of Trypanosoma cruzi in the vectors (Brener, 1973; Ribeiro et al., 1977 ; Lacombe, 1980; Lacombe and Dos Santos, 1984). Lacombe's evidence supporting the intracellular stage of T. cruzi in Triatoma infestans and Panstrongylus megistus is not convincing, due to the quality of the micrographs. In yet another study, Tobie (1968) injected bloodform parasites into the hemocoel of R. prolixus and found that T. cruzi could only survive there for a limited period. This would present a more convincing argument against the existence of hemocyte forms of T. cruzi in the insect if the insect forms of the parasite rather than the bloodform parasites had been injected into the hemocoel and had not lived.

Even though T. cruzi seems to be commensal with regard to the vector and no reports indicate any harmful effects of the parasite on the insect host, the environmental conditions of the insect must have some effect on the parasite's development (Brener and Alvarenga, 1976). This postulate seems plausible since the insect's environment may signal the parasites to transform and multiply.

Furthermore, it is not understood why certain Triatomine species are refractory to the parasite, or why differences in susceptibility exist even within a species (Phillips and Bertram, 1967). The host-parasite interaction has not been well-defined in these insects.

MATERIALS AND METHODS

The Vectors: Rhodnius prolixus and Triatoma infestans

Colonies of Triatoma infestans and Rhodnius prolixus, which have been maintained at Michigan State University since February of 1981, were raised in one liter Nalgene containers with nylon netted screw caps. Each container had several long W-shaped folded pieces of absorbent paper for egg attachment and absorption of fecal material. The insects were maintained in a dual program incubator at 28 °C and 50-60% relative humidity.

Every 7-21 days the insects were allowed to feed on defibrinated sheep blood through a latex membrane on an artificial feeding apparatus. The blood was kept at 37°C by the use of a pump which circulated water around it.

Whenever possible, fifth instar insects were used for experiments. Newly emerged insects were starved for 2-5 weeks before being allowed to feed, and only those that fed to repletion (approximately 60% of those fed) were used in these studies. Since the total hemocyte count of Rhodnius prolixus changes with the physiological state of the insect (Jones and Liu, 1961), it was desirable to use insects of

the same instar at the same time post feeding for experimentation.

Both Rhodnius prolixus and Triatoma infestans were fed on rabbits for many laboratory generations, beginning prior to their arrival at Michigan State University. In 1983, they were switched to sheep blood. The following experiments were conducted during a period of adaptation to this system. Many insects died after feeding or did not molt properly, so a stress factor was entered into the insect's normal physiological processes. Rhodnius prolixus was the primary species used at the onset of these studies. However, since Triatoma infestans seemed to adapt better to the new feeding system and more of these insects were available for study, it later became the principal subject.

The Parasite: Trypanosoma cruzi

Trypanosoma cruzi (Tulahuen strain) was maintained through passage in CD1 strain mice and cultured rat heart myoblast (RHM) cells. The Tulahuen strain was first isolated from Triatoma infestans in Chile in 1945 (Badinez, 1945; Taliaferro and Pizzi, 1955). Bloodform trypomastigotes (1×10^5) were injected through the intraperitoneal route into the mice, and infected blood collected 12-15 days later. The parasites were then purified and added to confluent RHM cells, which were maintained at 37 °C and 10% carbon dioxide in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum. While in culture with the cells under these same conditions, some of the

bloodform parasites enter the myoblasts, and transform into amastigotes. These amastigotes divide within the myoblast cells, and the cells eventually rupture, releasing trypomastigotes into the medium. At this point, three to five days after addition of the blood to the cells, trypomastigotes were removed with the medium, the medium centrifuged for 20 minutes at 1800 rpm, and the supernatant discarded. The parasites were either resuspended in DMEM and added to more RHM cells for further propagation, or resuspended in sheep blood (at least 1×10^7 parasites/ml) and fed to insects.

CHAPTER 3

THE ENCAPSULATION RESPONSE

INTRODUCTION

Examining the encapsulation responses of Trypanosoma cruzi-infected and non-infected Triatominae was the primary objective of this portion of research. An encapsulation response occurs when foreign bodies in an insect's hemocoel are too large to be engulfed by hemocytes. This response can be used as a measure of the immune reaction of the insect (Dawe et al., 1967; Salt, 1967; Nappi, 1973b). Often, natural parasites found within an insect's hemocoel do not produce an encapsulation response in the host, but it has been demonstrated that inert objects do (Salt, 1970).

In this study on the encapsulation response, nylon thread was used as the implant material. Initial encapsulation studies were performed with Rhodnius prolixus, but due to the small size of the R. prolixus colony and the difficulties in its maintenance, a different Triatomine species, Triatoma infestans, was used for the remaining experiments. The encapsulation responses of infected and non-infected T. infestans were observed and documented using scanning and transmission electron microscopy.

MATERIALS AND METHODS

Scanning Electron Microscopy (SEM)

Rhodnius prolixus and Triatoma infestans. Insects were allowed to feed on non-infected or T. cruzi-infected blood. At specified intervals post-feeding (see specific insect species below), insects were chilled on ice for 15 minutes to inhibit hemolymph coagulation (Rowley and Ratcliff, 1976). After localized surface sterilization of the insect with 70% ethanol (Horohov and Dunn, 1982), a nylon thread 150 micrometers in diameter and 500 micrometers long was inserted into the hemocoel perpendicular to the insect's exoskeleton on the right side of the third ventral abdominal segment. The thread had been surface sterilized in 70% ethanol and rinsed several times with sterile Insect Ringer's solution before insertion into the insect's hemocoel, according to the procedure of Bitkowska and coworkers (1982). The third ventral abdominal segment of the insect was chosen as the point of entry of the implant for the relative ease of insertion and removal.

The stiffness of the nylon thread facilitated insertion into the insect, and the size allowed for its relocation. Other items were also tested for implantation, and although all elicited an encapsulation response, consistency of technique was best using the nylon. Nerve cords from Blattella germanica were more difficult to insert, although their diameter was less; eggs from Delia antiqua were too large. Latex beads large enough to be encapsulated were inserted

readily, but were difficult to find thereafter. Different lengths and diameters of nylon thread were tried, and the one used for experimentation was the smallest possible while maintaining confidence in the technique. Although a smaller object would have been less disruptive to the insect's physiology, it was not practical experimentally.

At various times post insertion of the nylon (see below for specific species), insects were fixed by abdominal injection of cold 4% glutaraldehyde in 0.1M phosphate buffer, pH 7.2, after which the implant was carefully removed with forceps and placed in fresh buffered glutaraldehyde on ice for 1 hour. Most of the insects tested elicited an encapsulation response, but on rare occasion, the nylon thread became entangled in the fat body and was not encapsulated. These few samples were discarded. After fixation, the capsules were washed, dehydrated with an ethanol series, critical-point-dried, mounted on stubs, and sputter-coated with gold, according to the procedure of Hooper and co-workers (1979). An ISI Super III SEM was used at 15kV accelerating voltage to examine the specimens.

Rhodnius prolixus. Initial research was performed on this insect to determine when the encapsulation response in T. cruzi-infected and non-infected insects should be studied. These studies were done with only non-infected insects, using 4 insects per time point. Fifteen days after feeding, nylon threads were implanted into randomly-chosen insects, and then removed after either 0.25, 1, 24, 40 or 80

hours. Comparison of T. cruzi-infected and non-infected insects were performed on Triatoma infestans since more of these were available for study.

Triatoma infestans. Ten randomly-selected T. infestans were fed on infected blood and ten more on blood not containing T. cruzi. Fifteen and thirty days later, five of each were injected with pieces of nylon thread. The 15 day time point used in this study was also used by Bitkowska and coworkers (1982) in a similar study. Thirty days was also used to see if any differences found might change as the parasite progressed in its life cycle. Four hours after insertion of the thread, gut smears were taken from the T. cruzi-infected bugs to assure that an infection had, in fact, occurred, and the encapsulated implants were removed and processed for SEM as described previously. Initial studies had shown that 4 hours was ample time to observe an encapsulation response. Capsules were examined for differences in the amount of encapsulation and for surface structure changes on an ISI Super III SEM.

Transmission Electron Microscopy (TEM)

Since SEM did not reveal any significant differences in the encapsulation responses of infected and non-infected samples, TEM was used to determine whether the ultra-structure of the hemocyte-encapsulated nylon was changed.

Triatoma infestans which had fed on either T. cruzi-infected or non-infected blood were injected with nylon

thread either 15, 30, or 45 days post-feeding as previously described for SEM encapsulation studies, using a minimum of 3 insects per treatment. Twenty-four hours after insertion, the thread was removed from the hemocoel, post-fixed in buffered 1% osmium tetroxide, dehydrated with ethanol, including en bloc staining with uranyl acetate (Watson, 1958), embedded in Spurr's resin (Spurr, 1969), and polymerized. A Sorvall MT-2 ultramicrotome and a diamond knife were used to section the encapsulated material. Ultrathin sections were stained with Reynolds lead (Reynolds, 1963), then examined on a Philips 300 TEM operated at 80 kV.

The following criteria were used to compare the sections: 1. Was T. cruzi present? 2. How much material covered the nylon? 3. How were the layers structured? 4. Were the same types of cellular organelles present?

RESULTS

Scanning Electron Microscopy

Rhodnius prolixus. The progression of the encapsulation response to the nylon thread was observed in this study with non-parasitized Rhodnius prolixus. A nylon thread that was not inserted into an insect is shown for comparison (Figure 1). Within 15 minutes after insertion of the nylon into the hemocoel, hemocytes were attached to each other and to the thread (Figure 2). One hour post-insertion, few hemocytes retained their appearance as distinct cells (Figure 3). At this point, a substrate

PLATE 1

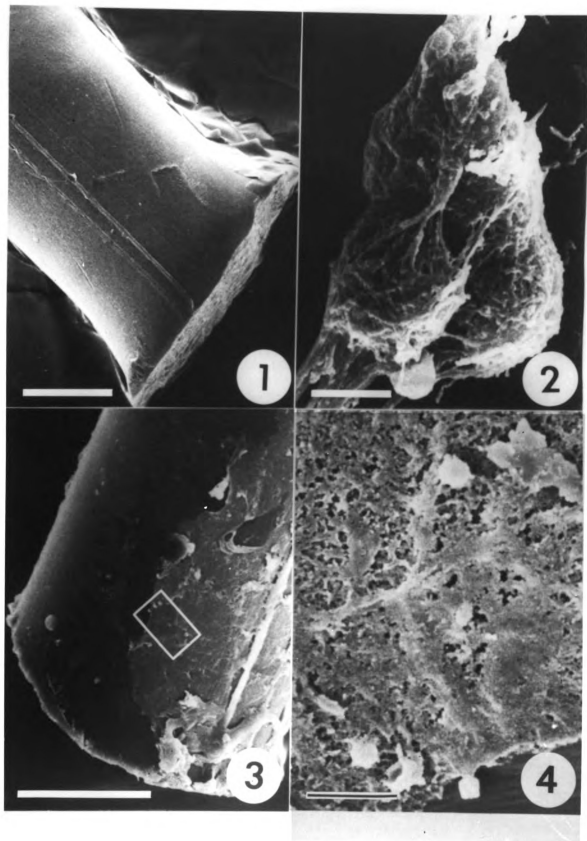
Figures 1-4 are scanning electron micrographs from the surfaces of nylon thread implants in non-infected Rhodnius prolixus.

Figure 1. Nylon thread prior to insertion. Bar equals 50 micrometers.

Figure 2. Surface of thread fifteen minutes after insertion into the hemocoel. Note the extensive filopodia of the attaching hemocytes. Bar equals 0.5 micrometers.

Figure 3. Surface of thread one hour after insertion. There is a thin coat of attaching material and few intact cells. Bar equals 50 micrometers.

Figure 4. Enlargement of the box in Figure 3, showing the extensive network of the coagulating material. Bar equals 0.5 micrometers.



consisting of a flat sheetlike network among the cells had formed (Figure 4). Encapsulation was generally sparse and encompassed only part of the thread, but melanization was already apparent under light microscopy.

The 24 and 40 hour capsules were larger as additional hemocytes joined the periphery of the already encapsulated thread and completely covered it (Figure 5). In addition, the encapsulating material now appeared more dense. The cells were attached to the thread and each other by filopodia which stretched out and thereby increased the surface area, while a noncellular substance filled in open spaces between the hemocytes (Figures 6 and 7).

After 80 hours inside the hemocoel, cells were still attached to the capsule (Figure 8), but not flattened out as seen earlier. Encapsulation seemed to be approaching completion, but this study was not carried out to the point of capsule termination due to the numbers of insects available.

Triatoma infestans. A capsule from a non-infected T. infestans was examined 15 days after feeding (Figure 9). The thread was completely covered by a smooth, dense material, thicker in some areas than in others. The encapsulation process was represented in a capsule taken from a T. cruzi-infected insect 15 days after the blood meal (Figure 10). Closely attached to the nylon thread was a thin filamentous layer similar to that seen in the early capsules of R. prolixus, followed by a thicker middle layer

PLATE 2

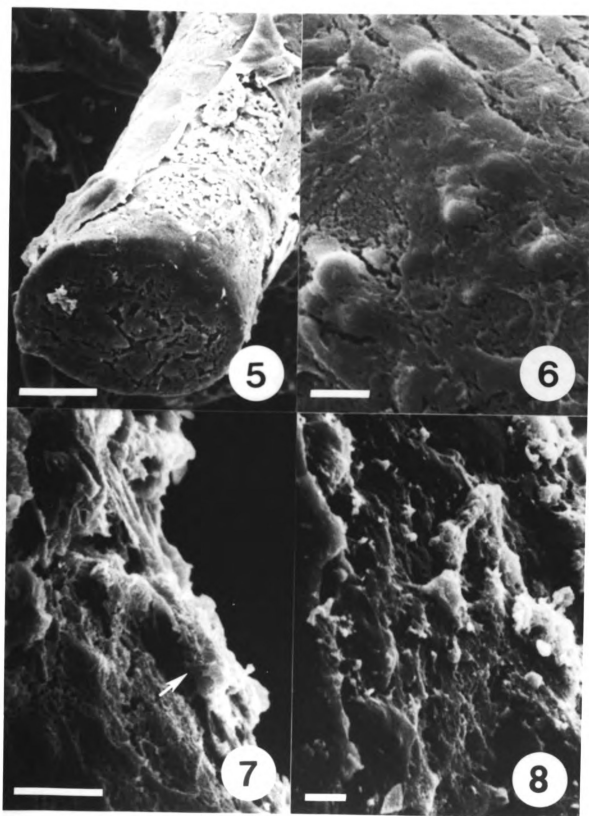
Figures 5-8 are scanning electron micrographs from the surfaces of nylon thread implants in non-infected Rhodnius prolixus. The nylon remained in the insect's hemocoel varying times.

Figure 5. Surface of a thread twenty-four hours after insertion. The thread is completely covered with a dense, seemingly non-cellular material. Bar equals 50 micrometers.

Figure 6. Surface of a thread forty hours after insertion. As in the twenty-four hour capsule, cells have attached and the spaces between them are filled with a substance similar to that seen in the earlier micrographs. Bar equals 1 micrometer.

Figure 7. Surface of a forty hour capsule. Note the hemocyte attaching and flattening out (arrow). Bar equals 1 micrometer.

Figure 8. Surface of an eighty hour capsule. The hemocytes attaching are not characterized by extreme flattening, marking near completion of the encapsulation response. Bar equals 1 micrometer.



with more hemocytes forming the outer layer.

A capsule from a non-infected T. infestans 30 days after feeding (Figure 11) appeared to be similar in morphology to non-infected and infected capsules at day 15. In this particular micrograph, however, more cells and debris were obvious. The tear in one portion of the material was most likely due to forceps and is not characteristic of the encapsulation. The encapsulated thread from a 30 day infected insect (Figure 12) appeared somewhat different in structure from the others. The surface appeared to be attached more loosely to the thread and more striated.

No consistent observable differences could be found between capsules from T. cruzi-infected or non-infected insects at either day 15 or day 30 after feeding (Figures 9 - 12). The amount of material covering a thread was variable within treatments, and the surface structure was similar in all specimens examined.

Transmission Electron Microscopy

Ultrathin cross-sections of the encapsulated nylon were taken from the end of the thread which was most medially located while implanted (Figure 13). The medial end was chosen for study as it generally showed more of an encapsulation response than the opposite end. The first sections taken from the medial portion of the capsule contained only cellular material, while successive sections included the nylon thread. Samples were compared according

PLATE 3

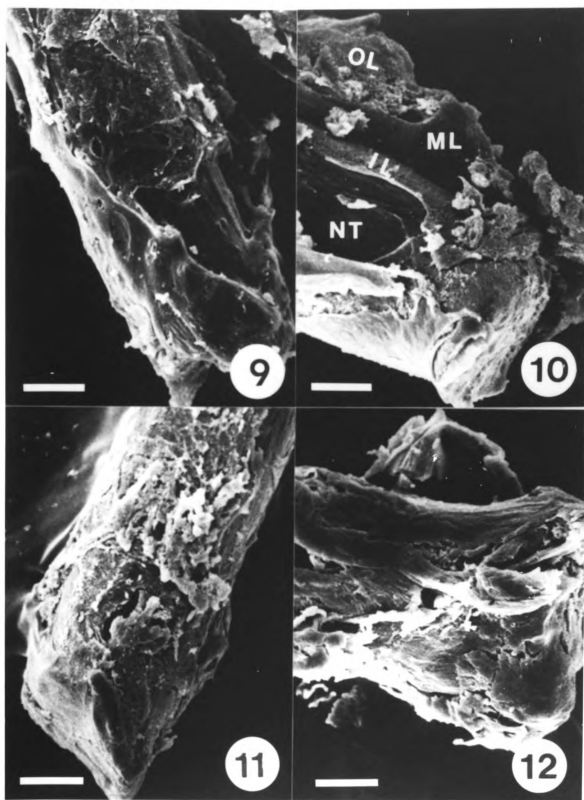
Figures 9-12 are scanning electron micrographs of four-hour capsules in Triatoma infestans.

Figure 9. Surface of a capsule from a non-infected insect fifteen days after feeding. Bar equals 25 micrometers.

Figure 10. Surface of a capsule from a Trypanosoma cruzi-infected insect fifteen days after feeding. Note the layers forming the capsule : NT = nylon thread, IL = inner layer, ML = middle layer, OL = outer layer. Bar equals 25 micrometers.

Figure 11. Surface of a capsule from a non-infected insect thirty days after feeding. Bar equals 25 micrometers.

Figure 12. Surface of a capsule from a T. cruzi-infected insect thirty days after feeding. Bar equals 25 micrometers.



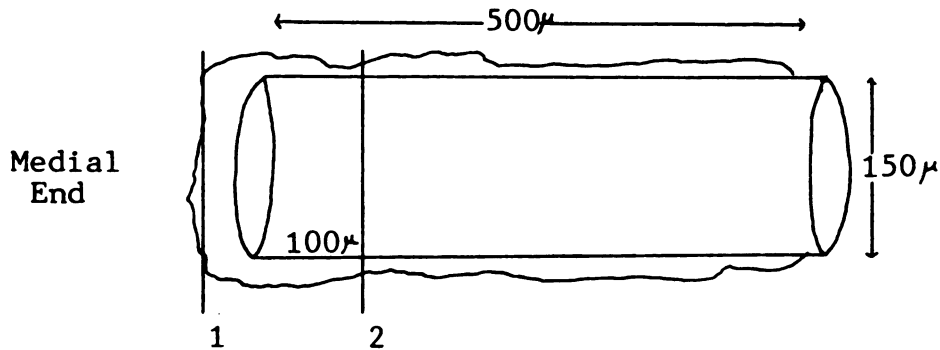


Figure 13. A diagram of a nylon thread which was implanted into *Triatoma infestans* and removed. The medial end, the end that was inserted deep into the hemocoel, shows more of an encapsulation response than the opposite end. Ultrathin cross sections were first taken at the medial end of the capsule (1) and did not include nylon. Consecutive sections were taken approximately 100 micrometers into the thread (2).

to the criteria stated earlier: 1. No T. cruzi were ever found within a capsule. 2. The amount of material encompassing the threads varied extensively in all samples, with the width in cross section ranging from 1-32 micrometers. 3. The general structure of the layers was similar regardless of the presence or absence of parasites or the day of insertion (Table 1). 4. Similar cellular organelles and hemocytes were found within the different capsules (Table 2). The quantities of these varied within groups and in different areas of the same capsule. Based on these results, no significant differences were found between the encapsulation response in T. cruzi-infected and non-infected insects, or between the capsules implanted at 15, 30, or 45 days after feeding.

The following is a general description of the structure of an encapsulated thread that had been in the hemocoel of Triatoma infestans for 24 hours. This applies for all the capsules studied, including both infected and non-infected insects.

In T. infestans, three layers encompassed the thread. The innermost layer generally consisted of an amorphous electron dense material believed to contain melanin, often including electron opaque inclusions and loose cellular components (Figures 14 and 15). The thickness of this layer varied extensively, and on occasion was not present or not electron dense, or was the only layer present.

When present in the capsule, a middle layer was found

Table 1. Characteristics of the layers which covered a nylon thread implanted into the hemocoel of Triatoma infestans

Number of Days after Insect Fed	Presence of <u>T. cruzi</u>	Number of samples surveyed	Number Displaying Characteristic M, C, N, L*												
			Inner layer				Middle layer				Outer layer				
			M	C	N	L	M	C	N	L	M	C	N	L	
15	-	3	3	0	0	0	0	0	2	0	1	1	2	0	0
	+	3	1	2	0	0	0	1	0	0	2	1	1	0	1
30	-	3	2	0	1	0	1	0	0	2	1	1	0	1	
	+	3	2	0	1	0	0	0	0	3	0	2	1	0	0
45	-	4	3	1	0	0	0	0	0	2	0	2	1	0	0
	+	4	2	2	0	0	0	1	0	2	1	2	2	0	0

^{*}M = melanized and electron dense, C = cellular material, N = necrotic cellular material, L = lacking.

Table 2. Presence (+) or absence (-) of cellular organelles in the hemocytic capsules of Triatoma infestans

Number of Days after Insect fed		Presence of <u>T. cruzi</u>	Number of Samples Surveyed	Number of samples positive (+) or negative (-)							
				Electron Dense Inclusions		Melanized		Lipids		Mitochondria	
				+	-	+	-	+	-	+	-
15	no		3	1	2	2	1	3	0	2	1
	yes		3	2	1	3	0	3	0	3	0
30	no		3	2	1	3	0	3	0	2	1
	yes		3	0	3	1	2	2	1	2	1
45	no		4	1	3	2	2	3	1	3	1
	yes		4	1	3	2	2	3	1	1	3

to contain flattened, necrotic cells, and an outer layer included many cellular organelles and open spaces (Figures 15 and 16). Cell membranes were seldom present, and the number of rows of hemocytes within a layer was variable. Non-melanized areas consisted of a disarray of cytoplasm, often containing long thick electron-dense areas (Figures 17 and 18). Occasionally, the outermost portion of the capsule was surrounded by a layer of melanin (Figures 18 and 19), and in one sample, bacteria were found in abundance (Figure 16).

The medial ends of the same capsules at the point not yet containing thread contained highly vacuolated lysing cells, with nuclei, membranes, mitochondria, and endoplasmic reticulum prominent (Figures 19-21). Lipid-containing cells (Figures 20 and 21), possibly attached fat body, polysomes (Figure 22), and electron dense areas (Figure 22) were also apparent in this part of the capsule.

DISCUSSION

The SEM studies on Rhodnius prolixus showed that the encapsulation of foreign material is a complex multicellular process in which hemocytes aggregate and segregate foreign bodies from the hemolymph. This reaction is not restricted to parasites in the hemocoel since inert objects, such as the nylon used in this study, also induce encapsulation.

The noncellular material which bonds the hemocytes to the thread, and the extensive stretching and adhesion of the

PLATE 4

Figures 14-16 are transmission electron micrographs of cross-sections from encapsulated twenty-four hour nylon threads in Triatoma infestans. Since no differences were found between T. cruzi-infected and non-infected insects, these are representative of capsules from either.

Figure 14. Inner layer (IL) which is melanized, electron-dense, and contains electron-opaque inclusions (arrow) and cellular remnants. IL = inner layer, NT = nylon thread. Taken from a T. cruzi-infected insect with the thread inserted 15 days after feeding. Bar equals 20 micrometers.

Figure 15. Electron-dense and compact inner layer (IL). Outer layer (OL) with a disarray of cellular components. N = nucleus, NT = nylon thread. Taken from a non-infected insect with thread inserted 15 days after feeding. Bar equals 20 micrometers.

Figure 16. Three layers of an encapsulated thread. The inner layer (IL) has electron-dense areas, the middle layer (ML) is clearly separated and makes up only a small portion of the capsule, and the outer layer (OL) consists of various cells which are loosely attached. Note the good preservation of the nuclei (N), but the general absence of cellular membranes. Bacteria (B) are also present in this sample. Taken from a T. cruzi-infected insect with thread inserted 15 days after feeding. Bar equals 20 micrometers.

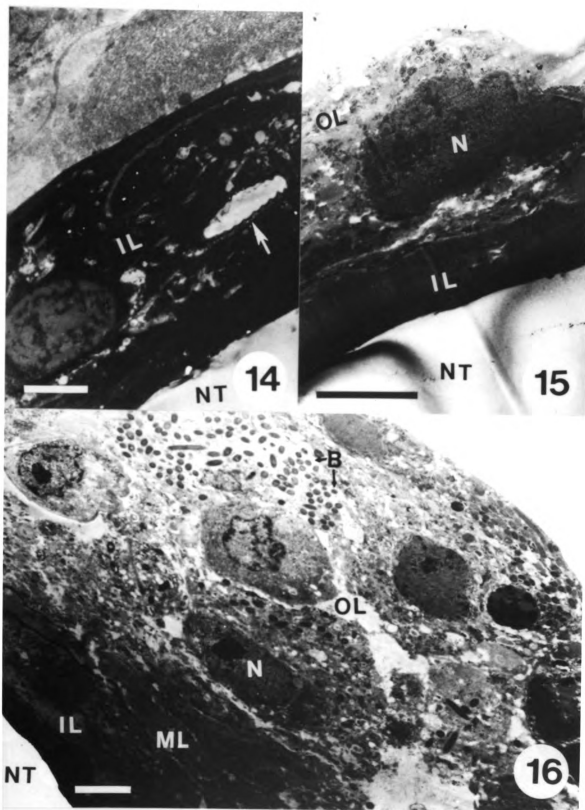


PLATE 5

Figures 17-19 are transmission electron micrographs of cross-sections from twenty-four hour capsules implanted in Triatoma infestans. These are representative of either T. cruzi-infected or non-infected insects.

Figure 17. Outer layer of a capsule consisting of lysed cells. Note the long threadlike electron-dense inclusion (arrow) characteristic of this layer. Taken from a T. cruzi-infected insect with thread inserted 15 days after feeding. Bar equals 20 micrometers.

Figure 18. Outer portion of a capsule with the characteristic array of cytoplasm that also contains a smooth electron-dense area believed to contain melanin (M). Taken from a non-infected insect with thread inserted 30 days after feeding. Bar equals 10 micrometers.

Figure 19. A capsule showing an extensive outer melanized layer (M). Taken from a non-infected insect with thread inserted 15 days after feeding. Bar equals 20 micrometers.

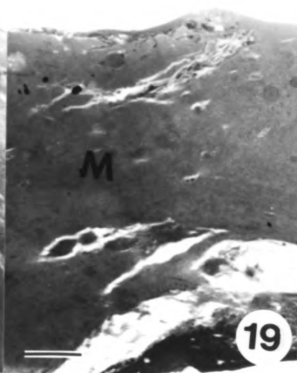
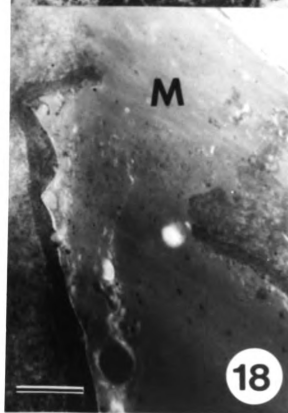
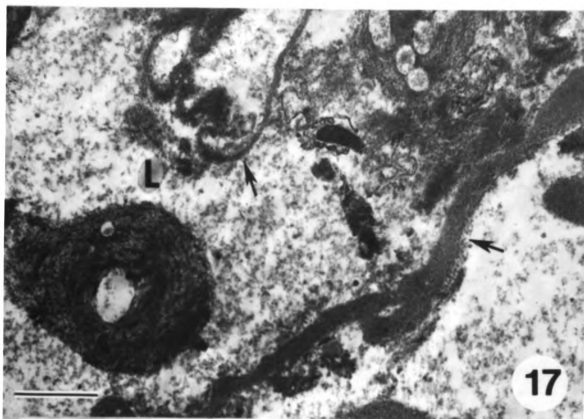


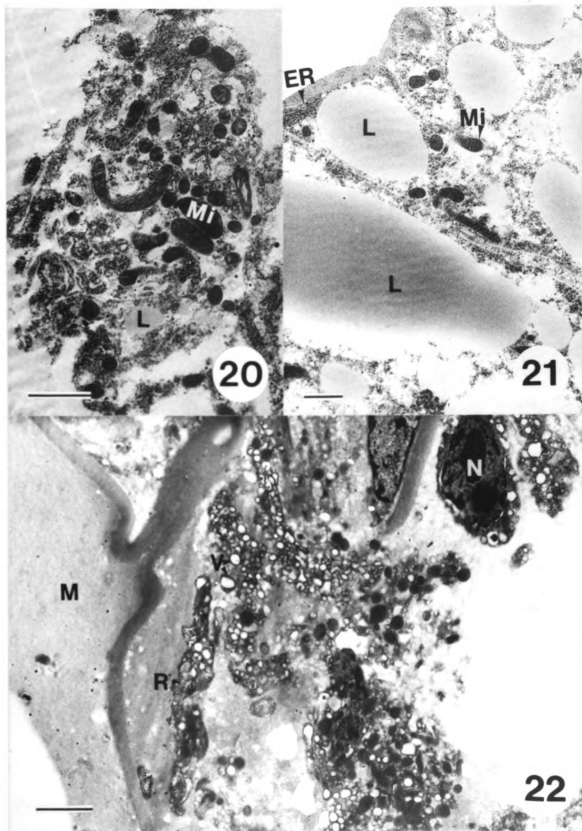
PLATE 6

Figures 20-22 are transmission electron micrographs of cross-sections taken from the medial end of a twenty-four hour capsule in Triatoma infestans. These are representative of either T. cruzi-infected or non-infected insects.

Figure 20. Loose cellular organelles. Mi = mitochondria, L = lipid. Taken from a non-infected insect with thread inserted 15 days after feeding. Bar equals 20 micrometers.

Figure 21. Prominent lipid inclusions (L), mitochondria (Mi), and endoplasmic reticulum (ER). Taken from a T. cruzi-infected insect, and the thread was inserted 30 days after feeding. Bar equals 20 micrometers.

Figure 22. Note dense threadlike structure similar to that of the outer layer, electron-dense melanized area (M), and lack of complete cells. Extensive vacuolization (V), nuclei (N), and polyribosomes (R) are all apparent. Taken from a T. cruzi-infected insect with thread inserted 15 days after feeding. Bar equals 20 micrometers.



hemocytes to form capsules is likely a result of mucopolysaccharide secretions and hemolymph coagulation. The flattening of the hemocytes also contributes to their ability to adhere and cohere (Nappi, 1975). By 80 hours, once the implant is well-encapsulated, this process is no longer apparent, an indication that encapsulation is nearing completion.

When areas were encompassed by cellular material, they had also undergone melanization. For Rhodnius prolixus, melanization is a principal event of encapsulation since melanization occurs within the first hour after insertion of the implant, and remains visible throughout the reaction. It is believed that this process, a result of the enzyme tyrosinase acting on tyrosine, will smother parasites, thus killing them (Nappi, 1978).

The work of Bitkowska et al. (1982) suggested that the equivalent of immunosuppression occurs in Trypanosoma cruzi-infected Triatoma infestans. This did not hold true in these SEM studies using the same insect species but a different strain of T. cruzi. There are several possible explanations: Genetics of the insect and/or parasite; induced stress; different techniques used in this study; or a combination of these.

Genetics and stress were already discussed in Chapter Two. Technical differences might have included the point of entry of the nylon thread, the implant material itself, the amount of time the implant remained within the hemocoel, and

the determination of what is considered a capsule. In both my investigation and that of Bitkowska and coworkers (1982), implants were inserted into the hemocoel, but Bitkowska did not reveal the point of entry. Bitkowska inserted tick organs and metal wire for xenografts, while I used nylon; however, they found no differences between the responses to tick organs and metal. I also implanted cockroach nerve cords, and observed a similar reaction to that of nylon. My four hour time point may not have allowed ample time to observe changes in the encapsulation response between T. cruzi-infected and non-infected T. infestans, but even at the 48 hour time Bitkowska used, encapsulation was not complete in these insects. In my studies, fatty tissue not firmly attached to the capsule was teased away for observation of only hemocytic reactions. Bitkowska did not state whether or not this was done in her work. However, results should be consistent for each investigator, since the techniques used were equivalent in T. cruzi-infected and non-infected insects, so may not explain differences in our results. Therefore, I believe that either genetics or stress must be responsible for the differences rather than technical difficulties.

The TEM ultrastructural encapsulation studies revealed no observable differences in the cellular defense responses of T. cruzi-infected and non-infected insects. Gut parasites have not been reported to exert an effect on the hemocytic responses of other insects that have been studied

(Nappi, personal communication). Although Lacombe and Santos (1984) reported an intracellular parasitic stage of T. cruzi in the Triatominae, their explanations are not conclusive. In my studies, no parasites (recognized by the 9 + 2 basal body of the flagellum) were found to have been incorporated into the hemocytes which had encapsulated the nylon thread, possibly implying that intracellular stages of T. cruzi do not exist. Another possibility is that hemocytes which have encountered parasites are not capable of performing both phagocytosis and encapsulation, or that parasites which were phagocytized may have undergone lysis and therefore are no longer visible.

Since encapsulation reactions in insects are known to involve intra- and extracellular deposition of melanin (Salt, 1970), the presence of melanin on the surface of the thread was not surprising. The variation of these encapsulated threads may have been enhanced by the recent change in diet of these insects, with some insects adapting better to the sheep blood than others. This additional stress variable makes it difficult to determine if effects and changes are due to the presence of the parasite or other physiological processes that occurred. The size of the thread, although the smallest possible that could maintain confidence in the technique, may also have contributed to the variation, since the whole thread was not always encapsulated. Portions of the encapsulated thread may have exhibited different stages of development, some nearing

completion and some far from it. It is possible that many of the hemocytes near the exoskeleton acted to close the wound rather than to encapsulate that portion of the thread. This finding is further qualified by the work of Salt (1961), who demonstrated that the wound healing process is similar to the encapsulation response in that the hemocytes aggregate and form a coagulant material which once again separates the hemocoel from the outside world. If the surface area of the xenograft had been less, there might have been more uniformity along the length of the thread.

Overall, encapsulation in Triatoma infestans is a complicated cellular process in which cells aggregate to form capsules around foreign bodies, melanin is formed, and localized hemocytes and hemolymph coagulate. In general, the innermost layer is pigmented and electron dense with cellular inclusions, followed by a layer of flattened cells and an outer layer of rounded cells. This is similar to the encapsulation reaction of most insects, in which the surface of an implant is covered by a heterogeneous, electron dense pigment layer, followed by flattened hemocytes, then normal ones (Nappi, 1975).

CHAPTER 4

HEMOCYTES

INTRODUCTION

Hemocytes, the cellular components of hemolymph in insects, not only encapsulate foreign objects, but also reveal infections in other ways. The number of hemocytes circulating within an insect as well as the percentage of the hemocyte types often varies with physiological state and may change during infection (Salt, 1970). Other effects of parasites on their insect hosts may be exhibited through surface or ultrastructural changes visible through electron microscopy. Tanaka (1982) showed by scanning electron microscopy that the hemocytes of braconid-parasitized noctuid moths exhibit shortened filopodia. To study the effect of parasites on the hemocytes' phagocytic ability, quantitative in vitro studies can also be performed.

Since encapsulation studies did not reveal obvious differences between Trypanosoma cruzi-infected and non-infected Triatoma infestans, it was hypothesized that any effects T. cruzi might have on the cellular immune response of its insect vector might be reflected in some other fashion by the hemocytes. Therefore, differential and total hemocyte counts were made on infected and non-infected insects, and in vitro assays of hemocyte phagocytosis were developed. To determine whether hemocytes in parasitized T. infestans and non-parasitized T. infestans were different,

scanning and transmission electron microscopy were utilized.

MATERIALS AND METHODS

Light microscopy

Hemocyte counts. Fed fifth instar Triatoma infestans were separated into 2 groups each containing 5 T. cruzi-infected and 5 non-infected insects. One group was used 15 days and the other 30 days after feeding. Hemolymph was collected by chilling each insect on ice for 15 minutes to reduce hemolymph coagulation, followed by removing a hind leg and bleeding the insect until the flow of hemolymph stopped. Rebleeding insects was attempted to increase sample sizes, however, at 30 days there was a visible change in hemocyte composition between those bled previously on day 15 and those not yet bled. Therefore, the hemolymph from each insect was examined separately and used only once for hemocyte counts.

Five microliter hemolymph samples were added to twenty microliters of a 2% saline-versene solution for total hemocyte counts. Four 1-mm squares in each chamber of a Neubauer hemocytometer were counted and the total counts calculated. The Mann-Whitney "U" test, a rank-sum test, was used for statistical analysis of differential and total hemocyte counts. The following formula was used for total hemocyte counts:

THC =

hemocytes in n 1-mm squares X dilution X depth of chamber
n

Differential counts were made through wet mounts of the remaining hemolymph. At least 200 randomly-selected hemocytes were counted per insect at 1000X with a phase-contrast microscope. The following key was used to differentiate the cell types:

1. Cells quite large; cytoplasm agranular, thick and homogeneous, generally with needlelike inclusions ... Oenocytoid (OE)
- 1' Cells variable in size; cytoplasm granular or agranular, without needlelike inclusions ... 2
2. Nucleus compact, large in relation to cell size, nearly filling the cell ... Prohemocyte (PH)
- 2' Nucleus not compact, not nearly filling the cell ... 3
3. Cells often pleomorphic and/or vacuolated, usually with filopodia and large dark inclusions which may obscure nucleus ... Plasmatocyte (PL)
- 3' Cells with thin homogeneous cytoplasm containing many fine granules, never with filopodia ... Granular hemocyte (GH)

In vitro hemolymph studies. Twenty-four adult T. infestans, unable to completely shed their old exoskeletons and not suitable for breeding, were used for this experiment. They were bled by severing a hind leg, and the hemolymph was placed into a cold Eppendorf tube which contained crystals of 1-phenyl-2-thiourea to prevent hemolymph melanization. Fifteen microliters of the pooled hemolymph were added to 3-mm wells in Teflon-coated slides

for either 5, 15, 30 or 60 minutes, since the time necessary for hemocytes to attach to the glass without detaching or lysing was not known. Samples were maintained either at room temperature or 37°C, since the insects were routinely maintained at 28 °C, close to room temperature, and a 37 degree incubator was available. Humidity was maintained at a high level in the incubator through a water-filled reservoir, but was not monitored for those samples at room temperature. After hemocyte incubation for the allotted period of time, the slides were washed with Insect Ringer's solution three times. Washing was essential to reduce the amount of hemolymph which coagulated. Then ten microliters of latex beads, 0.8 mm in diameter (1:100 dilution), in Dulbecco's Modified Eagle's Medium, was added to the cells (Wirth and Kierszenbaum, 1984). After 30 minutes, unattached beads were washed off. Cells were fixed in 4% glutaraldehyde, pH 7.2, washed, and stained with Giemsa. Differential counts were taken of 200 randomly-selected cells in duplicate or triplicate wells, and the number of cells phagocytizing latex particles counted. The number of cells per ten microscope fields (250x) was counted and averaged to determine whether attachment was dependent on time.

Scanning Electron Microscopy

Hemolymph from 3 non-infected Triatoma infestans and 3 with a 30 day T. cruzi infection was placed on round coverslips in a petri dish lined with moist paper.

Hemolymph samples were not pooled.

Several techniques were tried previous to this experiment for doing scanning electron microscopy on hemocytes: 1. A cover slip was coated with 1% poly-L-lysine to aid attachment (Macomber and Flegler, personal communication), 2. Hemolymph was placed directly onto the coverslip, allowing the cells to attach themselves. 3. One-phenyl-two-thiourea, an inhibitor of coagulation, was used, in combination with either poly-L-lysine-coated or non-coated coverslips. Allowing the cells to attach themselves gave the best results, so for these studies the hemolymph was left for 5 minutes on the slide in a petri dish lined with moist paper, excess fluid was removed and the coverslips washed gently with 0.9% NaCl, pH 7.2. The hemocytes were then fixed for 30 minutes in 4% buffered glutaraldehyde, pH 7.2, and dehydrated with a graded ethanol series. Samples were critical-point-dried using carbon dioxide as the transitional fluid, mounted on stubs, and sputter-coated with gold before viewing on an ISI Super III SEM. Identification of the hemocytes was based on comparison with light microscopy studies since few SEM studies have been done. At the time this research was initiated, Akai and Sato (1973; 1976) performed some of the only SEM investigations on the hemocytes of Bombyx mori, but Lepidopteran hemocytes are quite distinct from those of Triatoma infestans. All samples were observed for surface structure changes in the various hemocyte types and for the

presence or absence of T. cruzi.

Transmission Electron Microscopy

The following techniques were used: 1. Insects were bled directly into agar, then the hemocytes fixed; 2. Hemocytes were centrifuged, the pellet embedded in agar, then fixed; 3. Hemocytes were bled directly into the fixative. The final concentration of agar was 2% whenever used. Specimens were fixed in either 2 or 4% glutaraldehyde in 0.1M phosphate buffer, pH 7.2, for 1 hour. Two per cent glutaraldehyde was tried once to see if the concentration of the fixative might be too high, but since it made no perceptible difference, four per cent was used for all remaining experiments. After the primary fix, samples were washed twice, and post-fixed in 1% buffered osmium tetroxide for 90 minutes. Dehydration followed and included en bloc staining with uranyl acetate (Watson, 1958) at 50 and 75% ethanol. Ethanol was gradually replaced with acetone, then the samples were infiltrated with acetone/resin mixtures. A 50:50 Spurr:Epon 812 resin mixture was used for embedding (Hooper et al., 1979). Ultrathin sections were cut on a Sorvall MT-2 ultramicrotome using glass knives and post-stained in Reynolds lead (Reynolds, 1963). Samples were then viewed on a Philips 201 TEM operated at 60 kV.

RESULTS

Light microscopy

Hemocyte counts. There were no significant differences ($p < 0.05$) in differential hemocyte counts between infected and control Triatoma infestans on either day 15 or day 30, as shown in Table 1. (See Appendix A for individual numbers.) Plasmatocytes were the most numerous hemocyte, followed by granular hemocytes, prohemocytes, and oenocytoids.

At day 15 after feeding, the range of the total hemocyte count (THC) in non-infected T. infestans was 373-1012 cells/mm³, with a mean of 742, and in T. cruzi-infected insects, the THC ranged from 201-1600 cells/mm³, with a mean of 1124. At day 30, non-infected insects had THCs ranging from 445-1175 hemocytes/mm³, with a mean of 799, and the THC from T. cruzi-infected insects ranged from 275-3232 cells/mm³, with a mean of 1549.

In vitro studies. Those hemocytes which were allowed to attach for 5 or 15 minutes at 37°C with high humidity resembled fresh cell preparations, and the hemocytes incubated under the same conditions but for 30 or 60 minutes prior to addition of the latex beads had lysed. The numbers of cells per field for those samples which were maintained at 37 degrees were as follows: At 5 minutes, 34.7 +/- 2.2 cells were counted per field, 52.0 +/- 3.0 at 15 minutes, 41.0 +/- 3.0 at 30 minutes, and 48.0 +/- 6.0 at 60 minutes. Since hemolymph dried out in those samples kept at room

Table 1. Differential hemocyte counts of non-infected and Trypanasoma cruzi-infected Triatoma infestans

Days post Feeding	Presence of <u>T. cruzi</u>	Cell type ^a			
		Prohemocyte	Plasmatocyte	Granulocyte	Oenocytoid
15	-	7.0+/-0.3	69.0+/-1.1	23.0+/-1.5	0.6+/-0.4
	+	11.2+/-0.8	57.2+/-1.4	31.2+/-3.6	0.2+/-0.2
30	-	2.0+/-0.5	68.7+/-3.8	27.0+/-4.2	2.2+/-0.4
	+	1.4+/-0.5	62.6+/-4.2	34.0+/-4.1	1.8+/-0.6

^aAll counts are given as mean+/- standard error.

temperature with uncontrolled humidity, the few cells that attached were rounded, and counts were not taken.

Differential counts and latex bead phagocytosis were measured only in those hemocytes which had been allowed to attach for 5 or 15 minutes at 37 degrees (Tables 2 and 3), for only those cells appeared undisturbed by their environment. (See Appendices B and C for individual counts.) At 15 minutes, a larger number of granular hemocytes were phagocytizing latex than at 5 minutes, but the remaining data were not significantly different.

Scanning Electron Microscopy

No parasites were visible in the hemolymph of the T. cruzi-infected insects even though an infection was present in the digestive tract. The surface structures of hemocytes from infected and non-infected T. infestans were similar (Figures 1-4).

Granular hemocytes, prohemocytes, and plasmatocytes were all found in the hemolymph of T. infestans. The oenocytoid, making up only a very small percentage of the hemocyte population, was not observed on the SEM. Granular hemocytes (Figures 1 and 3) were approximately 9 micrometers long and were flat and oblong with small bumps and craters on their surfaces. The granular hemocytes of both T. cruzi-infected and non-infected insects which had adhered directly to the coverslip tended to attach on their ends and form a crescent shaped cell (Figure 1). The granular hemocyte shown in Figure 3 was found on top of another

Table 2. Differential hemocyte counts of in vitro populations from Triatoma infestans after different attachment times

Time	Hemocyte counts ^a			
	Prohemocyte	Plasmatocyte	Granular Hemocyte	Oenocytoid
5 min.	12.3+/-1.9	77.7+/-2.9	10.0+/-2.1	-
15 min.	24.0+/-8.5	63.5+/-7.4	12.5+/-1.5	-

^aAll counts are given as mean +/- standard error.

Table 3. Percentage of latex-associated hemocytes

Time	Hemocyte type ^a		
	Plasmatocyte	Granular hemocyte	All types
5 min.	66.7+/-3.5	48.0+/-4.4	64.3+/-3.5
15 min.	73.5+/-8.5	65.0+/-1.0	72.0+/-7.0

^aAll counts are given as mean+/-standard error.

PLATE 1

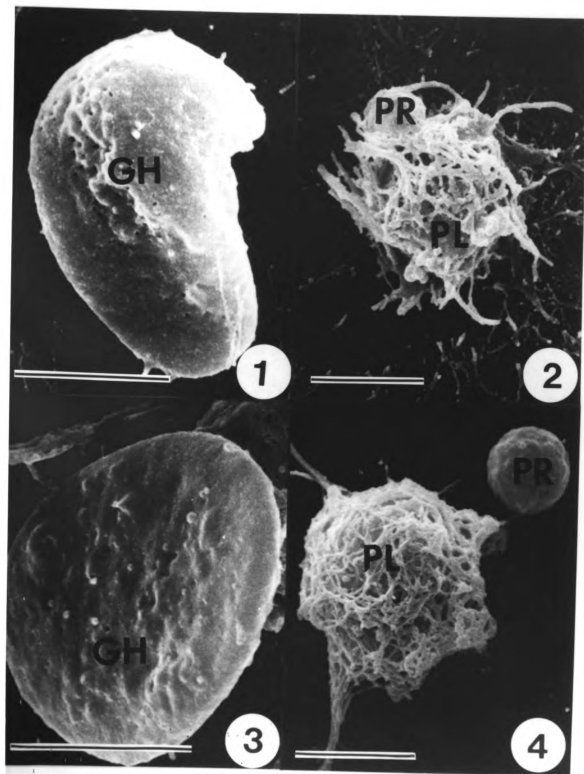
Figures 1-4 are scanning electron micrographs of hemocytes from non-infected and Trypanosoma cruzi-infected Triatoma infestans.

Figure 1. Granular hemocyte (GH) from a non-infected Triatoma infestans. The cell is attached to the glass coverslip. Bar equals 5 micrometers.

Figure 2. Prohemocyte (PR) and plasmastocyte (PL) from a non-infected T. infestans. Note the filopodia of the plasmastocyte. Bar equals 5 micrometers.

Figure 3. Granular hemocyte (GH) from a Trypanosoma cruzi-infected Triatoma infestans. The cell is not attached to the coverslip, and therefore maintains its flattened appearance. Bar equals 5 micrometers.

Figure 4. Prohemocyte (PR) and plasmastocyte (PL) from a T. cruzi-infected T. infestans. Bar equals 5 micrometers.



hemocyte, and did not adhere to the glass.

Plasmatocytes (Figures 2 and 4) were similar in size to granular hemocytes, but appeared more rounded, and had an extensive filamentous network of filopodia. Varying amounts of filopodial extension were exhibited among the hemocytes on a single coverslip. Prohemocytes (Figures 3 and 4), 1-3 micrometers in diameter, were generally rounded cells with small bulbous protrusions.

Transmission Electron Microscopy

Micrographs were never obtained, due to the few numbers of hemocytes per block and cell lysis during fixation.

DISCUSSION

Although not significant at the 0.05 level, a trend suggests that the numbers of plasmatocytes decrease and granular hemocytes increase during a T. cruzi infection (Table 1). Both of these cells are important in the immune response. Plasmatocytes are the major phagocytic cells while granular hemocytes produce coagulating material. However, since variability in both total and differential hemocyte counts was extensive, it is difficult to draw conclusions from the data. A possible explanation for the variability is the small sample sizes, and the use of the null hypothesis. Since the result is close to rejection of the null hypothesis on day 15, a one-sided hypothesis (i.e., the numbers of plasmatocytes decreases during infection), in combination with larger sample sizes, might be a worthwhile

pursuit.

The results from the in vitro studies should be interpreted with caution, as the T. infestans used were not representative of the insect population since the specimens had not molted properly. At both 5 and 15 minutes, the majority of the hemocyte population was made up of plasmatocytes (Table 2). At 15 minutes, the percentage of hemocytes that were plasmatocytes was less than it was at 5 minutes. A hypothesis which might explain this is that the plasmatocytes attach most readily, and by 15 minutes, more granular hemocytes and prohemocytes have attached, thereby increasing their proportions in the population.

Cell fields were counted to determine which attachment time acquired the most hemocytes, but it was not known whether a representative portion of the hemocytes had attached to the glass. A method to examine this would be to take an initial hemocyte count per microliter of hemolymph. Then it could be ascertained whether the population of cells on the slide adequately represented the insect's hemocyte population.

A possible explanation as to why fewer hemocytes had lysed and more were attached for the 30 and 60 minute time points is that a nutrient medium was not provided. For longer time periods the diet would be more essential. Later studies revealed that attached cells did not dry out at room temperature if provided with adequate humidity, explaining why the hemocytes had not fared well at the ambient

temperature.

A larger percentage of granular hemocytes phagocytized latex beads when allowed to attach for 15 minutes rather than 5 minutes (Table 3). A possible explanation is that since more cells had attached at 15 minutes than at 5, the proportion of cells to latex particles was larger, and therefore, the probability of a latex bead being proximal to a hemocyte was also larger. Another hypothesis is that cells needed more recovery time after attachment in order to be capable of phagocytosis, so more may have recovered at a 15 minute time rather than a 5 minute time. With more time and insects, in vitro phagocytosis could be studied using hemocytes of infected and non-infected insects to determine whether they respond differently.

No differences in the surface morphology of hemocytes were apparent on the SEM thirty days post-infection. One possible explanation is that the parasite, in the digestive tract, has no effect on the hemocytes, in the hemocoel. Earlier times post-infection might reveal differences, but due to the numbers of insects available, this was not performed.

Many techniques were used to attain ultrastructural information regarding the hemocytes. However, since the insects had few hemocytes, there were few insects, and hemocytes tended to undergo lysis, other projects were given priority over this one.

CHAPTER 5
ELECTROPHORETIC ANALYSIS OF HEMOLYMPH PROTEINS

INTRODUCTION

Hemolymph, a rich dynamic fluid high in free amino acids, supplies insect tissues with nutrients and removes their waste products, providing insects with a "buffer from the outside world" (Wyatt, 1975). Changes which occur in an insect's hemolymph during infection or parasitism can be used to learn more about that insect's defense response.

It was thought that analysis of hemolymph proteins of triatomine bugs with and without T. cruzi infections might reveal differences in protein composition. Although T. cruzi is a gut parasite, an ionic gradient is constantly maintained between the midgut and the hemolymph. As the hemolymph volume increases after feeding, nutrients from the bloodmeal enter the hemolymph through the Malpighian tubules. Since this exchange does occur, the parasite's presence in the gut might affect the protein contents or protein concentrations present in the hemolymph.

If such differences were apparent, this could provide an alternative means for xenodiagnosis, a method used to detect Chagas' disease. In xenodiagnosis, noninfected triatomines are fed on a suspected individual, the insects then incubated for approximately 30 days and their feces examined for the parasite. Since xenodiagnosis does not always reveal the presence of the parasite, serological

tests may also be required. In the rural areas where the disease is endemic, facilities and equipment for these serological tests are seldom available. An improved diagnostic technique would have wide applications, so if the protein content of the hemolymph were notably different in infected and non-infected insects shortly after feeding, it would save time in disease diagnosis and would have a higher diagnostic success. Uninfected insects could be fed on individuals as before and the hemolymph run on gels shortly after feeding to determine whether infection had in fact occurred.

MATERIALS AND METHODS

The species and numbers of insects used in the following experiments were based on availability. After insects had fed on blood either with or without T. cruzi, hemolymph samples were collected through a slit in the abdomen and micro-centrifuged to remove the cellular material in the hemolymph.

All electrophoresis techniques implemented follow those of Laemmli (1970), unless otherwise stated. Cell-free hemolymph was diluted 1:2 in sample buffer and boiled for 5 minutes. Standards, bovine serum albumin (m.w. 68K), ovalbumin (m.w. 43K), and concanavalin A (m.w. 26K subunits), were also prepared in this fashion. Samples were separated in one dimension on 8 or 10% sodium dodecyl sulphate (SDS) polyacrylamide slab gels with 4% stacking

gels, and then stained with 0.1% Coomassie Brilliant Blue in 75% acetic acid with 20% ethanol. Proteins were separated on 3 separate occasions.

Experiment 1

Four fifth instar Triatoma infestans which had fed approximately 30 days earlier, 2 on T. cruzi-infected blood and 2 on non-infected blood, were used for this experiment. Since less than five microliters of hemolymph was obtained from each insect and the protein concentration was not known, hemolymph from 2 insects was pooled to obtain adequate protein per sample. Fifteen microliters of each hemolymph preparation, one each from infected and non-infected insects, were run on wells of an 8% SDS gel.

Experiment 2

Two infected T. infestans, one an adult female and the other a fifth instar, were used. The controls included 4 insects: 1 adult female, 1 fifth instar which had recently fed, and 2 fourth instars. One of the fourth instars could hardly move and appeared to be dying. Hemolymph from each insect was used separately, since the runs in Experiment 1 indicated ample protein for this procedure would be present in each individual. Fifteen microliters of each diluted hemolymph preparation were run on an 8% SDS gel.

Experiment 3

Rhodnius prolixus was the subject of this experiment. Thirty non-infected and thirty T. cruzi-infected insects

were used. On days 7, 15, and 30 after the bloodmeal, 4 insects from each group were bled. All samples were frozen until analyzed. Hemolymph from every 2 insects was pooled to obtain adequate protein for electrophoresis since five microliters of hemolymph were needed for protein determination, and Rhodnius prolixus yields less hemolymph than does Triatoma infestans.

After all the hemolymph samples had been collected, protein concentration was determined according to the method of Lowry and coworkers (1951). Two hundred micrograms of protein of each sample were used per well of a 10% SDS gel. A cleaner separation of low molecular weight compounds was desired than that of previous experiments, therefore a higher gel concentration was necessary. To enable closer molecular weight approximations, additional standards (obtained from Bio-Rad) to those used previously included phosphatidyl B (m.w. 92.5K), beta-galactosidase (116.25K), and myosin (200K). After Coomassie blue staining, the gel was silver stained by the method of Wray et al. (1981).

RESULTS

Experiment 1

This first attempt at finding differences looked quite promising (Figure 1). A thin protein band, around 170K molecular weight and another relatively thick band about 75K appeared only in the well containing infected hemolymph. Also, several low molecular weight peptides appeared to

PLATE 1

Figures 1 and 2 are sodium dodecyl sulfate (SDS) - polyacrylamide gels of hemolymph proteins from Trypanosoma cruzi infected and non-infected Triatoma infestans. Standards (S) show molecular weights of 68,000, 43,000, and 26,000.

Figure 1. Gel of hemolymph proteins from 2 Trypanosoma cruzi-infected Triatoma infestans (T) and 2 control (non-infected) insects (C).

Figure 2. Gel showing hemolymph proteins from T. cruzi-infected T. infestans (lanes 4 and 6) and non-infected T. infestans (lanes 1, 2, 3, and 5). Fourth instars were used in lanes 1 and 3, but the one in lane one was dying. Fifth instars were used in lanes 2 and 4, and the one in lane 1 had fed recently. Lanes 5 and 6 were taken from adults.

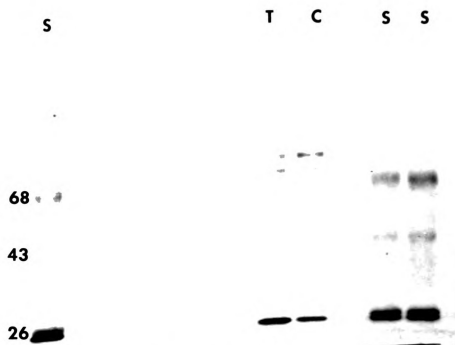


Figure 1

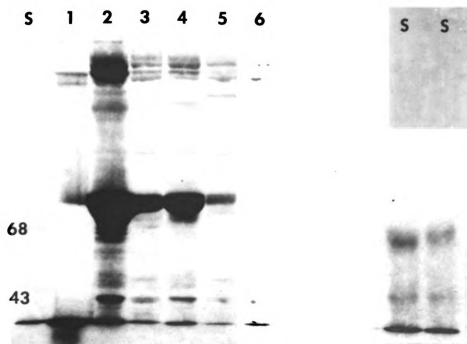


Figure 2

either be absent or in lesser quantities in the control.

Experiment 2

See Figure 2. Hemolymph from a dying fourth instar, in column 1, contained little protein. On the other hand, hemolymph from a fifth instar recently fed, in column 2, contained much protein, with many bands overlapping. Neither of these insects could be considered as a good basis for comparisons since the protein concentrations of the hemolymph samples were so different from the others. Comparison of hemolymph proteins from T. cruzi-infected insects (columns 4 and 5) with those from non-infected ones (columns 3 and 6) did not reveal the 170K protein found in the infected R. prolixus in experiment 1. However, the protein band around 90K was darker in the hemolymph from the infected insects, indicating more protein.

Experiment 3

See Figures 3 and 4. Some of the lower molecular weight material may be different, but the bands were not sharp or consistent enough to define changes.

DISCUSSION

The first experiment performed did reveal protein differences in infected and non-infected T. infestans. However, because it included only one run of each treatment, repetition was essential. It became apparent after the second experiment that the physiological state of the insect

PLATE 2

Figures 3 and 4 are sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) runs of hemolymph proteins from Rhodnius prolixus. Hemolymph samples were taken at days 7, 15, and 30 after feeding and 2 non-infected controls (C) and 2 Trypanosoma cruzi-infected (T) runs were taken each day. Standards (S) included a 200,000 and a 68,000 molecular weight protein.

Figure 3. Coomassie Blue stained gel of hemolymph proteins from infected and non-infected R. prolixus.

Figure 4. The same gel silver stained.

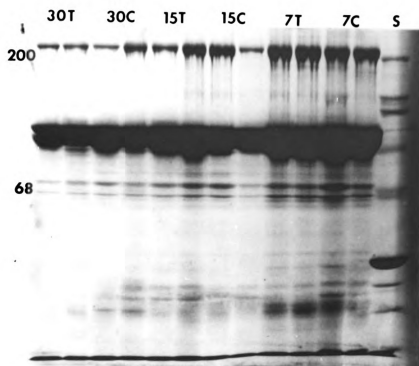


Figure 3

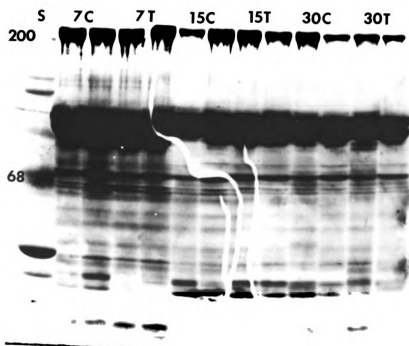


Figure 4

should be controlled to obtain interpretable results. More protein was found in the hemolymph of the insect that had recently fed, thereby overloading the gel, and the adults had fewer and different proteins from the nymphs.

In the third gel, the physiological state of the insect and the protein concentration were controlled, yet there were no observable differences in hemolymph proteins between infected and non-infected insects. Also, less protein should have been added to avoid overloading the gel.

There is not adequate evidence in these studies for the existence of differences in hemolymph proteins of triatomines that are infected or not infected with T. cruzi. Development of an alternative for xenodiagnosis, based on these results, was therefore not feasible.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The defense responses of non-infected and Trypanosoma cruzi-infected Rhodnius prolixus and Triatoma infestans were studied with the hope that this added information regarding the vector-parasite relationship might eventually lead to improved control measures for Chagas' disease. Research was conducted on the encapsulation response, hemocyte counts and morphology, and hemolymph proteins in both non-infected and T. cruzi-infected insects.

A piece of nylon thread was implanted into the hemocoel of R. prolixus or T. infestans to study the insect's encapsulation response. Rhodnius prolixus was used to determine the course of events in this response by observing the thread after up to 48 hours within the hemocoel. Through scanning electron microscopy, it was found that hemocytes aggregated together, flattened out, and attached to the nylon within the first hour. A substance, possibly the cytoplasmic contents of lysing cells, filled in spaces between the hemocytes. Eventually the thread was completely covered by coagulated hemocytes. The process of hemocyte attachment continued, increasing the amount of material covering the thread. After 48 hours within the hemocoel of the insect, cells were still attaching to the capsule, but were rounder than at earlier time points, possibly indicating the end of the encapsulation process. In T.

infestans, both scanning and transmission electron microscopy studies of the encapsulation response were conducted on insects either with or without a T. cruzi infection. There were no observable differences with either technique.

Another aspect of this research included hemocyte counts and morphology of infected and non-infected T. infestans. No significant differences were found between total hemocyte counts, differential counts, or scanning electron microscopy studies of individual hemocytes. Also, an assay was developed to study phagocytosis in vitro.

Hemolymph samples from infected and non-infected insects were studied by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Hemolymph proteins were separated on the basis of size, but no detectable differences were found.

Since no observable differences in the immune responses were found in T. cruzi-infected and non-infected T. infestans by any of the methods used, T. cruzi may not affect the insects' defense response. Trypanosoma cruzi, occurring in the digestive tract of its insect vectors, may evade the insect's defense response, which takes place in the hemocoel. More knowledge of the interactions of T. cruzi to its insect vectors is still necessary. A possible route might be to study the digestive processes and how they relate to parasite transformation and multiplication.

APPENDICES

APPENDIX A

Differential and Total Hemocyte Counts from Non-Infected
and Trypanosoma cruzi-Infected Triatoma infestans

The Mann-Whitney test is useful for data with non-normal distributions. The U statistic is defined by:

$$U_i = n_1 n_2 + \frac{n_i (n_i + 1)}{2} - R_i$$

where R_i equals the sum of ranks occupied by observations from a sample of size n_i , $i = 1, 2$. The null hypothesis is rejected that 2 individual random samples come from the same population when the value of U_1 is too small or too large.

Days after Feeding	Presence of <u>T. cruzi</u>	Differential Count*				Total Hemocyte Count
		PR	PL	GH	OE	
15	no	6	68	25	0	731
		7	72	19	2	769
		8	71	20	1	825
		7	66	27	0	373
		7	68	24	0	1021
	mean	—	—	—	—	—
		7	69	23	1	742
	yes	14	51	35	0	201
		10	71	17	1	1388
		10	54	36	0	1238
		12	56	32	0	1600
		10	54	36	0	1194
	mean	—	—	—	—	—
		11	57	31	0	1124
30	no	4	67	27	2	1175
		2	60	37	1	445
		2	73	22	3	1155
		1	81	14	3	725
		1	62	35	2	495
	mean	—	—	—	—	—
		2	69	27	2	799
	yes	2	53	45	0	3232
		1	64	33	2	1435
		3	55	38	3	1125
		1	64	34	1	275
		0	77	20	3	1675
	mean	—	—	—	—	—
		1	63	34	2	1549

*PR = prohemocyte, PL = plasmatocyte, GH = granular hemocyte, OE = oenocytoid.

APPENDIX B

Differential Hemocyte Counts in Cell Attachment Studies

Attachment Time	Sample Number	%Cell Type*		
		PR	PL	GH
5 minutes	1	16	73	11
	2	10	77	13
	3	11	83	6
		—	—	—
	mean	12.3	77.7	10
15 minutes	1	36	53	11
	2	12	74	14
		—	—	—
	mean	24	63.5	12.5

*PR = prohemocyte, PL = plasmatocyte, GH = granular hemocyte

APPENDIX C

Percentage of Latex-Associated Hemocytes

Attachment Time	Sample Number	% Latex-Associated Cells*	
		PL	GH
5 minutes	1	61	47
	2	73	56
	3	66	41
		—	—
	mean	66	48
15 minutes	1	82	64
	2	65	66
		—	—
	mean	73	65

*PL = plasmatocyte, GH = granular hemocyte

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