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INDUCED RESISTANCE TO IXODID TICK INFESTATION:  
ANALYSIS AND ISOLATION OF ANTIGENS

by

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Associate of Arts, Brainerd State Jr. College, 1969

Bachelor of Science, South Dakota State University, 1972

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A Dissertation

Submitted to the Graduate Faculty

of the

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for the degree of

Doctor of Philosophy

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Analysis and Isolation of Antigens

Department: Microbiology and Immunology

Degree: Doctor of Philosophy

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Induced resistance to ixodid tick infestation:  
analysis and isolation of antigens

By

Fred W. Olsen Jr.

Major, USAF, BSC

1987

Doctor of Philosophy Degree

University of North Dakota

ABSTRACT

Ticks are both vectors and reservoirs for significant pathogens of man and livestock. They are currently controlled through use of acaracides, but have rapidly developed resistance to every new acaracide produced to date. Alternative methods of control are needed. One proposed alternative is immunologic control, achieved through vaccinations which induce resistance to tick infestation. This proposal stems from the knowledge that cattle and laboratory animals actively acquire resistance to infestation. Immunization with crude extracts of different ixodid life cycle stages or organs has induced limited levels of resistance compared to acquired resistance. Defined tick antigens are needed to study the immunopathology and to develop vaccines. —>

Dermaacentor andersoni salivary gland antigen (SGA) and ova extract,

and Amblyomma americanum ova extract and gut antigens were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroeluted to nitrocellulose for immunoblotting. Antibodies from resistant guinea pigs and rabbits were used for immunodetection of immunogenic components in these fractionated tick extracts. Ultrafiltration and Sephadex gel filtration chromatography were used to fractionate A. americanum whole ova extract into defined molecular weight range fractions. These fractions were used in skin testing and immunization trials.

Skin testing elicited both immediate and delayed hypersensitivity reactions. Immunization with either the 30-100 Kdal fraction, prepared by ultrafiltration, or its gel filtration counter part, peak 2, induced a level of resistance to larval infestation that was comparable to that observed in acquired resistance. A component of gut antigen, 27,000 x g supernatant, induced a statistically significant level of resistance, in guinea pigs, to adult A. americanum. This resistance differs from acquired resistance in that the immunopathology occurs inside the tick and not in the skin of the host.

Hybridoma cell lines which produce monoclonal antibodies to D. andersoni ova extract and SGA were developed. Immunologic cross reactivity between A. americanum and D. andersoni was illustrated by immunoblotting, and cross resistance was demonstrated through sequential infestations. Molecules that contain cross reactive epitopes are candidate molecules to include in a vaccine because they will potentially broaden the coverage to multiple ixodid genera. The work presented here provides additional evidence that immunologic control of ticks is a realistic and achievable goal. *Stacy*

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## ABSTRACT

Ticks are both vectors and reservoirs for significant pathogens of man and livestock. They are currently controlled through use of acaracides, but have rapidly developed resistance to every new acaracide produced to date. Alternative methods of control are needed. One proposed alternative is immunologic control, achieved through vaccinations which induce resistance to tick infestation. This proposal stems from the knowledge that cattle and laboratory animals actively acquire resistance to infestation. Immunization with crude extracts of different ixodid life cycle stages or organs has induced limited levels of resistance compared to acquired resistance. Defined tick antigens are needed to study the immunopathology and to develop vaccines.

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## INTRODUCTION

### Tick Biology

#### Taxonomy

Taxonomically, ticks are Arachnida belonging to the order Acarina, superfamily Ixodiodea (Hoogstraal, 1978). Ticks are obligatory ectoparasites of vertebrates (Balashov, 1972). These biologically conserved hematophagous arthropods are divided into three families, the Ixodidae, or hard ticks, which includes 650 species, the Argasidae, or soft ticks, composed of 150 species, and the Nuttalliellidae with only one species (Hoogstraal, 1978).

#### Development and Feeding

All ixodids are obligate parasites of vertebrates. They are characterized by a complex developmental cycle (Balashov, 1972). They develop from an egg through three stages which are larva, nymph, and adult. Ticks take a blood meal, or engorge, once in each active instar or stage. Adult ixodids must engorge prior to mating and laying of eggs, or ovipositing (Balashov, 1972; Hoogstraal, 1978). Female ixodids oviposit only once in their life, but normally lay a large number of eggs (Balashov, 1972; Hoogstraal, 1978). This number varies by genus with Ixodes spp. laying 1,000-3,000 eggs (Balashov, 1984) on the low end, D. andersoni laying 5,000-6,000 eggs (Loomis, 1986) for a

mid range species, while on the upper end of the range, Amblyomma spp. lay 10,000-20,000 eggs (Balashov, 1984).

Attachment is the first stage of engorgement. Attachment begins with insertion of the chelicera or cutting mouth parts and the hypostome or hold fast organ into the skin of the host (Gregson, 1967). These mouth parts, in the case of Boophilus microplus, do not penetrate below the epidermis in any of the tick life stages and therefore rules out mechanical blood vessel damage as a factor in feeding as blood vessels are located in the dermis (Moorehouse and Tatchell, 1966). Tick salivary enzymes and host response mechanisms must therefore provide a blood and tissue fluid meal for these ticks. In other genera such as Ixodes and Amblyomma the long hypostome is inserted well into the dermis (Kemp et al., 1982; Moorehouse, 1967). Following insertion of the mouth parts, many ixodid species produce cement which forms a lamellate sheath around the hypostome and forms a cone to support and attach the external mouth parts to the host as well as isolate the tick mouth parts from the host (Moorehouse, 1967; Binnington and Kemp, 1980). Both species used in this research, Dermacentor andersoni and Amblyomma americanum, produce cement.

Ticks upon attachment may consume a small amount of blood from hemorrhage of superficial capillaries, but subsequently plasma, lysed tissue fluid, and infiltrating leukocytes constitute the main diet of all life stages (Tatchell, 1967; Balashov, 1972). Unlike vessel feeders, solenophages, such as the mosquito, which insert their fascicle into a capillary and take a rapid blood meal (Schmidt and Roberts, 1985), ixodid ticks are telmophages which feed slowly from a

pool, that develops beneath their mouth parts (Tatchell and Moorehouse, 1970; Kemp et al., 1982).

The body weight of an unengorged female ixodid tick increases by 80 to 120 times when it is fully engorged. Ixodid engorged body weight only indirectly reflects the actual ingested food volume, as much is excreted in the feces and excess water is removed via the salivary glands (Balashov, 1972; Binnington and Kemp, 1980). Bram (1975) reported that each Boophilus microplus tick consumes from one to three milliliters of blood in its life cycle. A typical host might be infested by thousands of Boophilus microplus. Horack et al. (1983) found that large ruminants in South African reserves were infested by multiple genera of ticks whose total numbers were in the thousands.

Although some of the more recently evolved ixodids are one host ticks, 600 of 650 species are three host ticks. Three host ticks generally infest a different host species for each of its life stages (Hoogstraal, 1978). A mode of feeding which is common for all ticks and consists of alternately sucking blood and lysed tissues, then injecting saliva into the bite site, has considerable ramification for the transmission and maintenance of disease causing organisms of man and domestic animals. A three host life cycle increases the possibilities of disease transmission and maintenance (Hoogstraal, 1978; Balashov, 1972; Gregson, 1967).

## Tick Host Interactions

### Impact of Infestation

Tick infestation and tick transmitted diseases are a serious problem throughout the world, but they exert the greatest impact in the tropic and subtropical regions (Bram, 1975; Wharton and Roulston, 1970). Ticks are the most important vectors of pathogens to wild and domestic animals and are second only to mosquitos in transmitting disease causing organisms to man (Balashov, 1972; Obenchain and Galun, 1982). They are truly equal opportunity vectors, transmitting bacterial, viral, rickettsial, and protozoan pathogens to their hosts (Balashov, 1972; Bram, 1975; Hoogstraal, 1981). Many of these pathogens have complex relationships with their tick vectors, undergoing development in various tick tissues and life cycle stages.

Pathogen transmission within the tick population occurs in two ways: (1) Transovarial transmission is from the adult female through the ova to the next generation. This insures the pathogen is transmitted to a large number of ticks, very few of which will successfully feed and further transmit the pathogen. (2) Transstadial transmission is from larva to nymph to adult. This makes ticks not only vectors, but also reservoirs for pathogens (Balashov, 1972; Hoogstraal, 1978; Binnington and Kemp, 1980; Solomon, 1983).

It has become apparent that man has intruded into well evolved relationships, when normally benign tick-borne infections of wildlife become serious diseases in domestic animals and man (Hoogstraal, 1981).

An example of this is babesiosis. Numerous species of Babesia, all transmitted by ticks, cause economically significant disease in domestic animals and have caused deaths in splenectomized humans. Nonsplenectomized humans may contract babesiosis which is usually characterized as an asymptomatic or mild illness (Hoogstraal, 1981; Hornick, 1983).

Some tick-borne infections have been known for hundreds of years while others have been "discovered" within the past decade (Hoogstraal, 1981). The epidemiology of tick-borne diseases is continually changing due to mobility of man and modification of environments (Hoogstraal, 1981). Examples which illustrate this are Rocky Mountain spotted fever caused by Rickettsia rickettsi, a rickettsial organism, and Lyme disease caused by Borrelia burgdorferi, a bacterial spirochete. Since 1930, a trend of increasing numbers of cases of Rocky Mountain spotted fever has been developing in the eastern United States (U.S.) while incidence has decreased in western states (Hoogstraal, 1981). Lyme disease, known for many years in Europe and Russia by various names, was first noted and identified in relation to an outbreak of suspected juvenile rheumatoid arthritis in the U.S. in 1975 (Hoogstraal, 1981; Hornick, 1983; Kujala, 1986).

Tables 1 and 2 are lists of some of the diseases ticks transmit to man and domestic animals. Epidemics of Crimean - Congo Hemorrhagic Fever (CCHF) occurred as early as 1100 A.D. Russian Spring-Summer encephalitis and CCHF are still causing significant morbidity and mortality in parts of the world (Hoogstraal, 1966). Infectious diseases can be spread by the bite of infected ticks as well as by

Table 1. Human diseases transmitted by ticks.

HUMAN TICK-BORNE DISEASES	AGENT	VECTOR GENERA
1 Erythema chronicum migrans	<u>Borrelia burgdorferi</u>	<u>Ixodes</u>
2 Lyme disease	<u>Borrelia burgdorferi</u>	<u>Ixodes</u>
3 Babesiosis	<u>Babesia microti</u>	<u>Ixodes</u> <u>Dermacentor</u>
4 Crimean-Congo hemorrhagic fever	Bunyaviridae	<u>Hyalomma</u> <u>Dermacentor</u> <u>Rhipicephalus</u>
5 Russian-spring-summer-encephalitis	Flavivirus	<u>Ixodes</u>
6 Tick-borne encephalitis	RSSE-TBE complex	<u>Ixodes</u>
7 Kyasanurforest disease	RSSE-TBE complex	<u>Haemaphysalis</u>
8 Powassan encephalitis	RSSE-TBE complex	<u>Haemaphysalis</u>
9 Colorado tick fever	Orbivirus	<u>Dermacentor</u>
10 Rocky Mountain spotted fever	<u>Rickettsia rickettsi</u>	<u>Dermacentor</u>
11 Boutonneuse fever	<u>Rickettsia conori</u>	<u>Dermacentor</u> <u>Ixodes</u>
12 Siberian tick fever	<u>Rickettsia siberica</u>	<u>Dermacentor</u> <u>Haemaphysalis</u> <u>Hyalomma</u>
13 Queensland tick fever	<u>Rickettsia australis</u>	<u>Ixodes</u>
14 American relapsing fever	<u>Borrelia spp.</u>	<u>Ornithodoros</u>
15 African relapsing fever	<u>Borrelia duttoni</u>	<u>Ornithodoros</u>
16 Asian relapsing fever	<u>Borrelia persica</u>	<u>Ornithodoros</u>
17 Q fever	<u>Coxiella burnetti</u>	<u>Amblyomma</u> <u>Dermacentor</u>
18 Tularemia	<u>Francisella tularensis</u>	<u>Dermacentor</u>

## Note:

Extracted from Hoogstraal, 1966; 1981.

RSSE-TBE complex = Russian-spring-summer-encephalitis and Tick-borne encephalitis viral complex.

Table 2. Diseases of cattle transmitted by ticks.

CATTLE DISEASES	AGENT	VECTOR GENERA
1 Babesiosis	<u>Babesia argentina</u> <u>Babesia bigemina</u>	<u>Boophilus</u> <u>Boophilus</u>
2 East Coast fever	<u>Theileria parva</u>	<u>Rhipicephalus</u>
3 Anaplasmosis	<u>Theileria annulata</u>	<u>Hyalomma</u>
4 Heartwater	<u>Cowdria ruminantium</u>	<u>Amblyomma</u>

## Note:

Extracted from: Bram, 1975; Uilenberg, 1976; and Loomis, 1986

their body fluids and feces which may contain pathogenic agents (Hornick, 1983; Needham, 1985).

Although ticks transmit diseases directly to man, resulting in morbidity and mortality, their impact on livestock production has greater ramifications for the world population. In many countries, tick-borne diseases such as East Coast Fever, Theileriosis, and Babesiosis are the major obstacle to efficient livestock production. These diseases result in substantially reduced amounts of meat, milk, and other animal products (Bram, 1975; Uilenberg, 1976; Steelman, 1976). Ticks and their pathogens cost livestock producers over \$275 million in lost productivity in the U.S. in 1985 ( Dr Ralph A. Bram, 1986 - personal communication). Costs for control of mites and ticks infesting beef and dairy cattle in the U.S. were estimated to be \$800 million (Loomis, 1986). These costs on a world wide basis are staggering (Bram, 1975).

Ticks impact livestock production in many ways. Although disease transmission is the most significant means, they also may cause: (1) anemia, due to the blood loss; (2) reduction in weight gain, due to worry and irritation; (3) damage to hides; (4) predisposition to secondary bacterial, fungal, or arthropod infections/infestations as a result of skin damage; and (5) paralysis with possible death, due to salivary toxins (Bram, 1975; Loomis, 1986; Solomon, 1983; Doube and Kemp, 1975).

## Tick Control

The need to control tick infestation and tick-borne diseases, due to their impact on animal health and the economics of livestock production, has been recognized for many years. The first chemical used as an acaricide, sodium arsenate, was used to eradicate Boophilus annulatus from the U.S. at the turn of the century (Solomon, 1983). Sodium arsenate was also used successfully to control ticks in South Africa and Australia from 1893 and 1895, respectively, to the mid 1930s, when resistance to this chemical was noted (Solomon, 1983). According to Durand (1976), Dr. R. H. Wharton reported that Queensland, Australia has the worst acaricide resistance problem in the world, with ticks having developed resistance to every type of chemical used to control them. Both the impact and the pattern of acaricide resistance in Africa and South America have been similar to that in Australia (Wharton and Roulston, 1970). Acaricide resistance is not universal even in a given area; however, once established, resistance appears to be stable (Wharton and Roulston, 1970; Solomon, 1983).

With the cost of producing and marketing a new acaricide exceeding \$10 million in 1976 (Durand, 1976) and possibly exceeding \$40 million in 1987 (Dr Stephen K. Wikel, 1987 - personal communication), the limited market and limited useful life expectancy has discouraged production of some new acaricides (Durand, 1976; Wharton and Roulston, 1970; Roulston, 1967). Virtually all tick control is currently based on the use of chemical acaricides. The rapid development of acaricide resistance points to the need to develop and apply alternative methods

of control which are less dependent on chemicals (Wharton and Roulston, 1970; Wikel, 1982c; 1983). An integrated tick management program including acaracides and alternative methods may provide better tick control and extend the useful life of currently employed acaracides (Wikel, 1984; Solomon, 1983).

An alternative, or adjunct method, to use of acaracides is artificial induction of host acquired resistance to infestation through immunization (Willadsen, 1980; Wikel, 1982; 1983; Wikel and Whelen, 1986). This approach to control requires a thorough understanding of the host immune response to ixodid feeding. Purified and defined immunogenic molecules of tick origin are needed for both analysis of host immune responses and immunization (Wikel and Whelen, 1986; Wikel et al., 1986; Gill et al., 1986).

### Bovine Immune Responses to Tick Infestation

#### Resistance

Johnston and Bancroft (1918) were the first to report naturally acquired resistance to infestation for cattle repeatedly infested with Boophilus microplus. Resistant cattle produced a lymph-like exudate at tick attachment sites, which trapped and killed the ticks (Johnston and Bancroft, 1918). A reduced fertility rate was noted for those adult females that engorged upon resistant hosts. It was proposed that this resistance developed in response to enzymes or other salivary components injected during tick feeding (Johnston and Bancroft, 1918).

Although most work with cattle has focused on the resistance developed to one host ticks of the genus Boophilus (Willadsen, 1980; Wikel, 1982; Wikel and Whelen, 1986), cattle have been reported to develop resistance to other ixodid ticks including; Ixodes holocyclus (Doube and Kemp, 1975), Haemaphysalis longicornis (Sutherst et al., 1979), Hyalomma anatolicum anatolicum (Gill, 1986), Dermacentor andersoni (Allen and Humphreys, 1979; Wikel and Osburn, 1982), and Amblyomma americanum (Brown et al., 1984b; George et al., 1985).

Resistance has been characterized by a full spectrum of tick responses including: simple rejection with little or no damage to the tick, reduced numbers of engorged ticks, reduced engorged weights, prolongation of feeding time, reduced numbers and viability of ova, and death while attached to the host (Willadsen, 1980; Wikel, 1983; 1984). This indicates that the resistant host's immune defenses may have a significant impact on the tick's life style and survival.

Resistance response of cattle appears to have a genetic basis (Wharton et al., 1970). Asian breeds, Bos indicus, were thought to be innately more resistant to tick infestation than European breeds, Bos taurus (Francis and Little, 1964; Wharton et al., 1970). Doubt was cast on the concept of innate resistance by the work of Wagland (1975; 1978), which indicated tick naive cattle of both breeds were equally susceptible to infestation. Willadsen (1980) indicated that the difference observed in the rate and degree by which resistance is attained may be expected interspecies variation; while Wikel (1983) suggested it may be a result of differences in bovine immune response gene expression. The latter would be consistent with the variability

in response of individual cattle, of the same breed, to immunization observed by Johnston et al. (1986).

### Immune Mechanisms of Bovine Host Resistance

#### Antibody

Cattle resistance to tick infestation involves a number of immune effector mechanisms (Willadsen, 1980; Wikel, 1982c). Homocytotropic antibody was shown to be stimulated in cattle by infestation with Boophilus microplus (Riek, 1956; 1962). Resistant cattle, injected intradermally with extracts of Boophilus microplus larvae or eggs, developed immediate hypersensitivity reactions. Presence of homocytotropic antibody to tick extracts was confirmed, by passively transferring cutaneous hypersensitivity from a tick resistant animal to a tick naive animal, as demonstrated by a positive Prausnitz-Kustner reaction (Riek, 1956; 1962).

Following infestation with Boophilus microplus, Brossard (1976) found serum gamma globulin levels were elevated. Both specific and nonspecific antibodies to tick salivary gland were detected using immunofluorescence. Specific antibodies are found only in infested animals, while nonspecific antibodies are also found in uninfested control animals. Passive transfer of resistance to Boophilus microplus with massive volumes of plasma taken from resistant calves and given to naive calves strongly supports a role for antibody in acquired bovine tick resistance (Roberts and Kerr, 1976). Wikel and Osburn (1982)

found that low level infestation of cattle with Dermacentor andersoni stimulated production of precipitating antibody to salivary gland antigen.

#### Cell Mediated Immune Response

There is limited information on the role of cell mediated immunity for the bovine-tick host relationships (Willadsen, 1980; Wikel, 1984). Wikel and Osburn (1982) were able to demonstrate cell mediated effector mechanism in cattle infested with Dermacentor andersoni. Animals with two or more low level infestations gave significant delayed type hypersensitivity skin reactions, characterized by induration, when tested with Dermacentor andersoni salivary gland antigen. Cross reactivity to other ixodid species salivary gland antigens was also observed in this skin testing (Wikel, 1984). As a further indication that a cell mediated response was involved, in vitro lymphocyte blastogenesis studies were performed using the same salivary gland antigen. The unfractionated peripheral blood lymphocytes from infested animals proliferated in a specific and dose dependent manner while those from unexposed controls did not proliferate (Wikel and Osburn, 1982). One can not say this is definitely a cell mediated response because the lymphocytes were unfractionated and proliferation could have been by T and/or B lymphocytes.

George et al. (1985) performed a similar study, but used low level infestations of Amblyomma americanum ticks. Skin testing gave delayed reactions to salivary gland antigens from Amblyomma americanum.

Amblyomma cajennense, and an immediate hypersensitivity reaction to Dermacentor andersoni SGA. In vitro lymphocyte blastogenesis studies showed that lymphocytes from infested animals proliferated in a specific and dose dependent manner to Amblyomma antigens (George et al., 1985). The delayed hypersensitivity reaction supports the theory of cell mediated mechanisms functioning in tick resistance. Since the lymphocyte blastogenesis studies were not performed using enriched subpopulations of lymphocytes, a cell mediated response, although indicated, is not confirmed. Proliferation may have been by either T and/or B-lymphocytes.

#### Histology of Bovine Tick Attachment Sites

The histology of cutaneous reactions to ixodid tick infestation in naive and resistant cattle has provided clues to host response mechanisms (Wikel, 1984). Binnington and Kemp (1980) pointed out the need for careful interpretation of histological findings as demonstrated by earlier interpretations attributing development of the feeding lesion to lytic salivary secretions. Enzymes such as phospholipase, proteinase, and hyaluronidase were shown to be absent from tick saliva, by histochemical technique, but enzymes such as esterases, phosphatases, and polyphenol oxidase were present (Tatchell, 1967). These enzymes may help create a small lesion and vascular damage to provide the tick a blood meal (Binnington and Kemp, 1980). The major tissue damage leading to development of the feeding lesion appears to be attributable to infiltration and degranulation of neutrophils leading to autolysis

(Tatchell and Moorhouse, 1970; Berenberg et al., 1972; Binnington and Kemp, 1980; Gill, 1986). Histology, which provides clues to host response mechanisms, needs to be combined with studies designed to characterize specific immune mechanisms in order to accurately interpret the role these mechanisms play in resistance to tick infestation. (Wikel, 1983).

Cattle resistant to Boophilus microplus were found to have intense dermal infiltrations of eosinophils, other polymorphonuclear leukocytes which were not identified further, and mononuclear cells at Boophilus microplus attachment sites. The early response was characterized by eosinophil infiltrate and correlated with immediate hypersensitivity to tick bite (Riek, 1962). Immediate hypersensitivity reactions might be protective because they result in avoidance behavior (Stebbing, 1974) such as increased grooming activities, which significantly reduce Boophilus microplus larvae within the first 24 hours (Willadsen, 1980). Larvae move and reattach but have little significant damage caused to them by the host response except removal through grooming during the first 24 hours of infestation (Willadsen, 1980).

Schleger et al. (1976) found that Boophilus microplus attachment sites on resistant cattle showed the presence of degranulating mast cells and an infiltration predominated by eosinophils by three hours post attachment. Neutrophils were noted and increased in numbers in five hour lesions. Basophils were seen in thin sections but were in low numbers; therefore, these cells were not quantitated. Later lesions involved infiltration of the epidermis with development of vesicles containing neutrophils, mononuclear cells, and degranulating

eosinophils. Unexposed hosts lacked mast cell degranulation and eosinophils at the attachment site, emphasizing the immunologic nature of the reaction (Schleger et al., 1976).

Allen et al. (1977) reported that Ixodes holocyclus elicited a cutaneous basophil hypersensitivity reaction in cattle previously exposed to that species. Brown et al. (1984b) found that bovine responses to repeated infestation with Amblyomma americanum were characterized histologically as a cutaneous basophil hypersensitivity reaction. Gill (1986) reported that cattle resistant to Hyalomma anatolicum anatolicum infestation responded to reinfestation with dermal infiltration of neutrophils, basophils, mononuclear cells (fibroblasts, lymphocytes, monocytes, and macrophages), and eosinophils in order of their numerical prevalence. Vesicles containing mainly neutrophils and a few basophils formed at every attachment site on resistant hosts, and serous exudate on the skin surface trapped some of the ticks, which died without engorging.

Because of the complex nature of tick resistance in cattle its exact composition and mode of action have not been fully defined. It is apparent that both humoral and cellular mechanisms are involved. There appear to be at least two mechanisms or two manifestations of the same mechanism of resistance occurring; one a hypersensitivity reaction which merely leads to tick removal and a second which directly affects the ticks ability to obtain an adequate blood meal and proceed with normal biologic functions (Willadsen, 1980). Basophils have been implicated in resistant host responses, but have not been reported in all cases. This may be due to histologic techniques used in earlier

studies. Use of controlled conditions and genetically defined laboratory animals may contribute significantly to the elaboration and understanding of tick resistance.

### Animal Immune Responses to Tick Infestation

#### Resistance

Trager (1939a) performed the first reported experimental studies of tick infestation of laboratory animals. Guinea pigs expressed acquired resistance to Derma-centor variabilis larvae after a single infestation of either larvae or nymphs. Subsequent infestations yielded fewer engorged larvae which also weighed less than those from a first infestation. Brown (1977) confirmed these findings for Derma-centor variabilis larvae on resistant guinea pigs, and it was additionally noted that there was a decreased number of larvae molting and an increase in the length of time for molting to occur. Allen and Kemp (1982) reported an increased frequency of detaching and reattaching of Derma-centor andersoni larvae on resistant guinea pigs compared to susceptible guinea pigs. Although some larvae died while still attached to resistant guinea pigs, most adversely affected larvae died after detaching from the host (Allen and Kemp, 1982). Trager (1939a; 1939b) pointed to the immunologic nature of tick resistance and proceeded to attempt to elucidate the mechanisms involved through experimentation using laboratory animals.

## Immune Mechanisms

Antibody Mediated Immune Response

Trager (1939a) demonstrated the role of antibody in tick immunity by passively transferring resistance with serum from guinea pigs resistant to D. variabilis to tick naive animals. Serum, from guinea pigs resistant to Ixodes holocyclus, passively transferred a limited resistance to tick naive guinea pigs which was characterized by a cutaneous basophil hypersensitivity response (Bagnall, 1975). Askenase et al. (1982) passively transferred a high level of resistance to Rhipicephalus appendiculatus with serum, but for Ixodes holocyclus resistance developed by passive transfer of serum was only modest.

Tick rejection by passively immunized guinea pigs was associated with cutaneous basophil reactions (Askenase et al., 1982). Efficacy of protection provided by passive transfer of resistance with serum may be affected by both the tick and host species as well as the route of administration (Askenase et al., 1982). Wikel and Allen (1976a) reported that resistance to Dermacentor andersoni could be passively transferred with viable lymph node cells but not with serum. Serum was administered with one intraperitoneal injection rather than by the intravenous route. A slight, but not statistically significant, decrease in larval engorgement weights was noted. Thus the route of administration of serum may be pertinent as Askenase et al. (1982) successfully transferred significant levels of resistance to tick infestation, with serum by the intravenous (IV) route, for two ixodid

species, Ixodes holocyclus and Rhipicephalus sanguineus. Working with Amblyomma americanum ticks, Brown (1982) passively transferred resistance to naive guinea pigs using serum, via the IV route, which reduced tick yield to 56% compared to naive hosts with an engorged tick yield of 75-81%.

Haynes et al. (1978) demonstrated that IV transfer of small amounts of guinea pig homocytotropic antibody, specifically IgG1, resulted in an antigen specific cutaneous basophil hypersensitivity (CBH) response in newborn guinea pigs. Local administration of IgG1 results only in passive cutaneous anaphylaxis (PCA), but systemic administration results in both PCA and CBH responses to antigen (Haynes et al., 1978; Askenase et al., 1979). Although the mechanism by which CBH is transferred systemically by guinea pig IgG1 is not known, properties which may contribute are: (1) cytophilic attachment to mast cells and basophils by its Fc portion, and (2) its ability to fix complement by the alternative pathway resulting in chemotactic factors for basophils being released (Haynes et al., 1978; Brown et al., 1982). Transfer of CBH by guinea pig IgG1, anti-keyhole-limpet hemocyanin (KLH), was blocked by prior administration of heterologous whole cytophilic IgG or Fc fragments but not by (Fab')<sub>2</sub> fragments (Graziano and Askenase, 1979).

Brossard et al. (1982) working with rabbit basophils and salivary gland antigen (SGA) from Ixodes ricinus found that basophils were progressively sensitized in vivo with each additional infestation as was demonstrated by in vitro degranulation assays. Lett-Brown et al. (1983) showed that basophils were sensitized in vitro with serum, from

guinea pigs that were immune to KLH and that responded to KLH with a CBH response. Passive transfer of serum, from guinea pigs immune to KLH to unimmunized guinea pigs, resulted in more efficient sensitization of circulating basophils than occurred in vitro. This was confirmed by in vitro basophil degranulation by homologous antigen (Lett-Brown et al., 1983).

These findings help explain the importance of the route for administering serum in regard to acquisition of resistance to tick infestation. The passive transfer of serum by the IV route appears to be more effective in transmitting resistance to tick infestation than administration by other routes. This may be due to in vivo sensitization of circulating cells such as basophils. In actively acquired resistance, where both antibody and cell mediated mechanisms are found functioning simultaneously, the level of resistance is greater than can be obtained by either passive transfer of resistance with serum or adoptive transfer of resistance with lymph node cells alone. This may be due to synergism between antibody and cell mediated mechanisms. Synergism could result in a greater degree of resistance than merely the sum of the two individual mechanisms.

Brossard et al. (1982) found an agent, thought to be IgE, which enhanced basophil sensitization and increased in activity with each successive infestation. Interestingly, a second agent, which blocked basophil sensitization, was found to also increase with each infestation. This blocking agent was speculated to be IgG, which might neutralize SGA (Brossard et al., 1982). It appears that what has been

described, two agents with opposite effects on basophil sensitization, may be the counter balancing arms of a regulatory system.

Boese (1974) demonstrated the presence of homocytotropic antibody by passive cutaneous anaphylaxis (PCA) in rabbits resistant to Haemaphysalis leporispalustris. The strength of the PCA reaction induced by the serum correlated directly with the degree of resistance observed in the donor rabbit. Precipitating antibodies were not demonstrated. McGowan (1985) reported a similar hypersensitivity mechanism of resistance in wild cottontail rabbits, Sylvilagus floridanus, but that the snowshoe hare, Lepus americanus, did not produce skin sensitizing antibody and did not develop resistance to infestation with Haemaphysalis leporispalustris. Resistance to Haemaphysalis longicornis, in rabbits, was characterized only by a reduction in engorged weight (Fujisaki, 1978). Fujisaki (1978) found precipitating antibodies of the IgG class in the sera of resistant rabbits but tests for homocytotropic antibody were not performed.

Brossard (1977) passively transferred resistance to Ixodes ricinus from a resistant rabbit to a susceptible rabbit with two intravenous injections of serum. Ticks infesting the immune serum recipient had engorgement weights which were 25% less than ticks from normal serum recipients. A greater degree of resistance was observed when a doubled volume of immune serum was injected into the recipient rabbit (Brossard and Girardin, 1979). The increased resistance of the serum recipients was characterized by a 29% reduction in engorgement weight and 39% fewer females oviposited, when compared to ticks from control rabbits. Both circulating IgG and homocytotropic antibodies, which were specific

for Ixodes ricinus antigens were identified in resistant rabbits (Brossard and Girardin, 1979).

Wikel and Allen (1976b) provided further evidence for the role of antibody by using cyclophosphamide which greatly depleted B-lymphocytes in resistant guinea pigs, thus suppressing antibody production and blocking resistance to infestations. It appears that antibody is a major factor in tick resistance. Since passive transfer results in animals with less resistance than the serum or plasma (antibody) donors which actively acquired resistance, it is apparent other mechanisms are also active in the host protective response (Wikel and Whelen, 1986).

#### Cell Mediated Immune Response

Bagnall (1975), using inbred guinea pigs, adoptively transferred resistance to Ixodes holocyclus with lymph node cells obtained from resistant guinea pigs to tick naive recipients. The recipients of immune host cells rejected 37.6 - 64.5% of a larval challenge. Wikel and Allen (1976) adoptively transferred a statistically significant ( $P < 0.001$ ) degree of resistance to Dermacentor andersoni with cervical lymph node cells from guinea pigs infested twice with 100 larvae. Resistance was expressed by decreased engorgement weights and reduced numbers engorging. Wikel et al. (1978) demonstrated cell mediated mechanisms were functioning in resistance to Dermacentor andersoni through skin testing and in vitro lymphocyte blastogenesis assays. Skin testing of resistant animals with salivary gland antigen elicited

strong delayed hypersensitivity reactions, while in vitro lymphocyte proliferation to SGA was antigen specific.

#### Histology of Tick Attachment Sites

Trager (1939a) found significant differences in the histology of Dermacentor variabilis larval attachment sites on naive and resistant guinea pigs. Attachment sites on naive guinea pigs had a small hemorrhagic area, but no cellular infiltrate in either the dermis or epidermis, nor any thickening, of the epidermis was evident. In resistant guinea pigs the picture was dramatically different with a large accumulation of leukocytes dominated by polymorphonuclear cells infiltrating the dermis. Infiltrate contained very few eosinophils. Epithelium at the edges of the reaction was thickened and appeared to be growing down around the leukocyte mass as to wall it off; and the whole area was edematous. The reaction described by Trager is currently called an epidermal bulla. Allen (1973) described similar lesions, but in contrast to Trager, found the dermis, of resistant guinea pigs reinfested with Dermacentor andersoni, to be infiltrated with a notable number of eosinophils. Through the use of thin sections large numbers of basophils were identified and the reaction bore a striking similarity to cutaneous basophil hypersensitivity.

The histology of tick attachment sites have shown basophils to be significant in guinea pig development of resistance to the following ixodid ticks: Dermacentor andersoni (Allen, 1973), Amblyomma americanum (Brown and Askenase, 1981), Ixodes holocyclus (Bagnall,

1975; Brown et al., 1984a), Rhipicephalus appendiculatus (McLaren et al., 1983), and Rhipicephalus sanguineus (Brown and Askenase, 1981).

Gordon and Allen (1979) monitored the numbers of basophils in the blood and bone marrow of guinea pigs during a primary and secondary infestation with Dermacentor andersoni larvae. Basophils increased in both the bone marrow and the blood. Peripheral blood basophil numbers increased on the fourth day post primary infestation. Basophil numbers were elevated the first day of the second infestation and continued to increase through day 18, or one day after the end of the second infestation and then decreased to normal levels. Increases in bone marrow basophils may have slightly preceded those observed in the peripheral blood. The increase correlated very closely to both the time and to the concentration of basophils found at attachment sites. A more rapid onset of blood basophilia occurred and basophils reached higher concentrations in secondary infestation, than were observed in a primary infestation. This is also what was seen histologically at tick attachment sites (Gordon and Allen, 1979).

Brown and Askenase (1982) found similar blood basophilia patterns in guinea pigs infested with Amblyomma americanum larvae. Unlike Gordon and Allen (1979) who found no change in the concentration of eosinophils, neutrophils, lymphocytes, or monocytes in either blood or bone marrow, Brown and Askenase (1982) reported peripheral blood eosinophilia which ran parallel with basophilia and correlated with increased eosinophils at tick attachment sites. This would be consistent with the earlier studies of the histology of Amblyomma americanum attachment sites performed by Brown and Knapp (1980a; 1980b) in which

eosinophils were the third most prominent cell found. Timing was very important in determining the dominant cell type in this dynamic process. Basophils were the dominant cell type at 12 hours post attachment in resistant guinea pigs, but by 72 hours post-attachment eosinophils were predominant (Brown and Knapp, 1981). Basophils have also been shown to be significant in the resistance of rabbits to Ixodes ricinus (Brossard and Fivaz, 1982) and Hyalomma anatolicum anatolicum (Gill and Walker, 1985). Additionally, as previously mentioned, basophils were shown to play a role in bovine resistance to Amblyomma americanum (Brown et al., 1984) and Hyalomma anatolicum anatolicum (Gill, 1986). Cutaneous basophil hypersensitivity appears to be a common host response to ixodid infestation (Wikel and Whelen, 1986).

Effector cells are very important in the acquisition and expression of resistance. Matsuda et al. (1985) found that mast cell deficient mice were unable to acquire resistance against H. longicornis larvae until bone marrow transplant replenished the deficient mast cells. These findings were contradicted by those of DenHollander and Allen (1985b) in which the same mast cell deficient strain of mice were reported to develop resistance, although not as strong as that of similar mast cell sufficient mice, to infestation by Dermacentor variabilis. As histological studies were not reported in this study, the only factor which can be commented on is that a possible species specific difference in the ticks may have resulted in resistance developing by a different route for Dermacentor variabilis, possibly basophils, thus by-passing the mast cell deficiency.

By administering antibasophil specific antibody to resistant guinea pigs, Brown et al. (1982) eliminated resistance to Amblyomma americanum infestation. A decrease in tissue eosinophils at the attachment site was noted and attributed to decreased eosinophil chemotact factor of anaphylaxis (ECFa) being released from basophils. Further, administration of specific anti-eosinophil antibody depleted the eosinophils leaving the number of basophils intact. This allowed the expression of resistance, but it was a weaker response than observed in controls. Thus the expression of resistance in the guinea pig - Amblyomma americanum system requires synergism between basophils and eosinophils (Brown et al, 1982; Dvorak et al., 1986).

Although cutaneous basophil hypersensitivity seems to be a dominant, if not universal response to ixodid tick infestation, several investigators did not find basophils in the systems they studied. DenHollander and Allen (1985a), examining the BALB/c mouse response to infestation with Dermacentor variabilis, and Matsuda et al. (1985), studying a mouse - Haemophysalis longicornis system, found resistance appeared to be associated with mast cells and eosinophils. Tatchell and Moorhouse (1970) reported finding neutrophils as the primary response in dogs infested with Dermacentor variabilis, but did not indicate either the type or quantity of other cells in the response. Rubaire-Akiiki and Mutinga (1980) found rabbits infested with Rhipicephalus appendiculatus had few mast cells and responded predominantly with eosinophils. Some investigators may not have observed basophils as they lose metachromatic staining properties after aqueous fixation and are not easily detected with ordinary histologic methods

(Dvorak et al., 1970). Rubaire-Akiiki and Mutinga (1980) used appropriate techniques to detect basophils, but found none. Possible reasons presented for not finding basophils were that the basophils may have been disrupted in processing, or that the timing was such that a wave of basophils had degranulated bringing on a new wave of eosinophils. Willadsen (1980) and Gill (1986) speculated that the cellular response to tick infestation of resistant hosts may be species specific and dependent on the particular combination of host and parasite.

Langerhans cells (LC) are dendritic, ATPase-positive, suprabasal cells of the epidermis with the proposed function of trapping antigenic material in the epidermis and presenting it to immunologically competent cells in a manner similar to macrophages (Allen et al., 1979a). Dermacentor andersoni salivary gland antigen (SGA) was found, by indirect immunofluorescence, to be trapped by LC at the dermal-epidermal junction of resistant guinea pigs but not in an initial exposure (Allen et al., 1979b). Allen et al. (1979b) noted that complement, IgG, and SGA deposits were observed at the dermal-epidermal junction after day five of an initial infestation and every day of a secondary infestation, but in the latter case these substances were detected within one millimeter of the attachment site. Activation of complement by SGA and specific antibody through the alternative pathway could contribute to cell infiltration and creation of a lesion (Allen et al., 1979b). Nithiuthai and Allen (1984a) observed that the numbers of LC decreased significantly around tick attachment sites in primary infestations and increased during secondary infestations in a similar manner observed in sensitization and challenge in contact hypersen-

sitivity. Langerhans cells were shown to be able to act as antigen presenting cells in guinea pig immune response to tick infestation (Nithiuthai and Allen, 1985) and this decrease in LC at attachment sites may be due to LC migrating to draining lymphnodes during primary infestation (Dr. John R. Allen, 1987 - personal communication). Morphologic changes occurred in LC during second infestations and function appeared to change from antigen presenting to one of antigen laden target. As such the LC did not migrate, but became the focus of humoral and cellular immune reactions (Nithiuthai and Allen, 1984a). Perhaps this merely reflected the action of pre-existing antibody and complement and/or cytotoxic T-cells acting before the cell could migrate. Treatment of guinea pig ears with short wave ultraviolet light (UVC) caused dramatic and prolonged decrease in normal epidermal LC lasting at least five days (Nithiuthai and Allen, 1984b). Both the acquisition and expression of tick resistance was significantly reduced in guinea pigs that had LC depleted by short wave ultraviolet light (Nithiuthai and Allen, 1984c).

#### Host Mediators Contributing to Tick Resistance

##### Complement

It is apparent from the studies already cited that tick resistance is an immunologic process involving both humoral and cellular components. Other immune response mediators have been shown to play significant roles in the development and expression of this very

complex reaction to tick infestation. Wikel and Allen (1977) evaluated the role of complement in both acquisition and expression of resistance. Complement levels in guinea pigs were depleted by 85-95% using cobra venom factor. This had no effect on the acquisition of resistance, but blocked the expression of resistance. The complement cleavage product, C5a an anaphylatoxin, has significant chemotactic and granulocyte-aggregating activity and the ability to activate intracellular functions in some cells resulting in release of oxygen metabolites and leukotrienes (Cooper, 1984). This may partially account for the basophil infiltration at tick feeding sites.

The alternate pathway of complement activation was shown to be important in the expression of tick resistance (Wikel, 1979). Guinea pigs that were C4-deficient, thus lacking a classical pathway of complement but possessing a functional alternative pathway, were able to acquire and express resistance to Dermacentor andersoni larvae at the same level as Hartley guinea pigs with no complement deficiencies. The resistance expressed was characterized by a CBH response and serous exudate which trapped and killed unengorged larvae.

#### Histamine

Histamine is a major component of mast cells and basophils and has been hypothesized to be involved in resistance to tick infestation (Willadsen, 1980; Wikel, 1982; Brown, 1985). In cattle resistant to Boophilus microplus elevated blood histamine levels were found by Riek (1962); while Willadsen et al. (1979) found elevated levels of his-

tamine in the skin. Wikel (1982) measured histamine levels at Dermacentor andersoni attachment sites on tick resistant guinea pigs. The level was significantly increased compared to the histamine content of similar sites on one exposure and control guinea pigs. Administration of type-1 and type-2 histamine receptor antagonists (antihistamines) individually had no effect on the expression of resistance; but, given concurrently reduced expression of resistance (Wikel, 1982). Using different antihistamines and doses, Brown and Askenase (1985) did not affect resistance expression and suggested other basophil mediators may be involved. Comparing these findings with the ones obtained by Wikel (1982) Brown and Askenase (1985) suggested that differences may be due to the wider range of effects promethazine has compared to mepyramine, including impacting functions of T-cells; or be a species specific difference such that histamine might not have a primary role in resistance to Amblyomma americanum, but have one for Dermacentor andersoni.

Paine et al. (1983) induced Dermacentor andersoni to feed on defibrinated sheep blood through a mouse skin membrane while they electrically monitored their feeding. Addition of 10 mM histamine and serotonin resulted in decreased feeding activities as demonstrated by decreased electrical amplitude and duration of the signal recorded on the chart of the electrical monitor. Eventually the ticks stopped feeding. DenHollander and Allen (1985a) noted Dermacentor variabilis fed more readily on males than on female BALB/c mice. The females had in excess of twice the level of histamine in both their dorsal and ventral skin when compared to males. Brossard and Fivaz (1982)

reported that histamine seemed to be involved in rabbits acquiring resistance to female Ixodes ricinus. This resistance could be inhibited by daily treatment with mepyramine, a type-1 histamine receptor antagonist. Bagnall (1975), using a type-1 antihistamine, induced a slight reduction in the resistance expressed by guinea pigs resistant to Ixodes holocyclus.

Kemp and Bourne (1980) found that histamine injected intradermally under tick larvae engorging in vivo caused ticks to detach. Histamine exerted its effect on ticks which had attached 16-24 hours earlier but resulted in very few ticks detaching after 48 hours of being attached. Additionally, once the tick larvae had been attached for three days, histamine had no effect on their weight gain. In the case of Boophilus microplus on cattle the most important function of histamine may be to cause larvae to detach frequently during the first day and cause irritation to the host resulting in increased grooming which facilitates larvae removal (Willadsen, 1980; Kemp and Bourne, 1980). The exact mechanisms by which histamine functions in tick resistance are unknown (Kemp and Bourne, 1980; Wikel, 1982).

#### Tick Alteration of Host Response

In the process of feeding, ticks inject salivary secretions into the bite site and these salivary substances stimulate host immune responses (Trager, 1939a; Wikel, 1983). One might expect a highly evolved, slow feeding, obligate ectoparasite to have developed mechanisms to facilitate engorgement and avoidance of host protective immune

responses (Wikel, 1985). Wikel (1982) was the first to report on immunosuppression associated with tick infestation. Lymph node cells from guinea pigs resistant to Dermacentor andersoni responded in vitro in an antigen specific manner to salivary gland antigen; but, showed marked reduction to T-cell mitogens, phytohemagglutinin and concanavalin-A. Response to B-cell mitogen, E. coli lipopolysaccharide was not affected (Wikel, 1982). Additional evidence that D. andersoni induce a transient T-cell immunosuppression was provided by assaying for IgM production to a thymus dependent antigen, sheep red blood cells. A direct hemolytic plaque-forming cell assay showed reduced numbers of cells producing IgM at the end of an initial infestation and during a second exposure with return to normal levels four days post infestation (Wikel, 1985). Impaired T-lymphocyte function could make the host more susceptible to tick-borne pathogens and facilitate feeding in the presence of host immunity (Wikel, 1982).

Ribeiro and Spielman (1986) found that saliva of Ixodes dammini inhibits anaphylatoxins (C3a and C5a), which are products of complement activation and important components of the hosts inflammatory response. Incubation of anaphylatoxins with tick saliva prevented the effects of anaphylatoxin both in inducing contractions of the guinea pig ileum and in increasing skin vascular permeability. Ixodes dammini saliva was also shown to contain agents which are antihemostatic, antiinflammatory, and immunosuppressive (Ribeiro et al., 1985). The antihemostatic activity effected both platelet aggregation and coagulation. Platelet aggregation is stimulated by adenosine diphosphate (ADP). Inhibition of platelet aggregation was found to be due to an apyrase,

which hydrolyzed both ADP and adenosine triphosphate (ATP) but not adenosine monophosphate (AMP), and prostaglandins of the E series, which increases platelet cyclic AMP levels. Coagulation time was extended due to the effect of a salivary component on the intrinsic pathway of coagulation. The prostaglandins (PGE<sub>2</sub>) immunosuppressive activity was demonstrated by its inhibitory activity on a cloned T-cell hybridoma resulting in suppression of interleukin-2 (IL-2) secretion. The presence of a kinase which hydrolyzes bradykinin, known to produce pain, explains how ticks are able to feed without causing pain (Ribeiro et al., 1985).

Chinery (1981) found pharmacodynamic components in the saliva of Rhipicephalus sanguineus sanguineus which were histamine like and caused vasodilation in naive rabbits, but had a greater reaction in sensitized rabbits, and thus indicated its antigenic nature. Having previously detected a histamine-blocking agent in the saliva of Rhipicephalus sanguineus sanguineus (Chinery and Ayitey-Smith, 1977), the discovery of this pharmacodynamic component of saliva led to the proposition that both salivary components were produced to regulate fluid availability for engorgement (Chinery, 1981).

Both Higgs et al. (1976) and Dickinson et al. (1976) isolated prostaglandins from the saliva of the cattle tick, Boophilus microplus. One function of prostaglandins is to increase vascular permeability and this may act to help regulate fluid supply for feeding (Dickinson et al., 1976).

Evaluation and Significance of Resistance

Resistance to tick infestation of laboratory animals has been evaluated using multiple parameters, such as number of ticks engorging, engorgement weight, viability of ticks recovered, length of feeding period, egg production, and percent of eggs hatching (Trager, 1939a; Willadsen, 1980; Wikel, 1983). Each of these parameters evaluates the effect of resistance on the tick. Wikel (1980) looked at an aspect which may be even more important, the effect resistance had on the transmission of pathogens. Only 36.4% of rabbits resistant to D. andersoni infestation were infected and died when fed on by D. andersoni carrying Francisella tularensis compared to 100% of the non-resistant controls. Francis and Little (1964) found Drought Master cattle, a Bos indicus and Bos taurus cross breed, were more resistant to tick infestation than Herefords. Only 11.3% of the tick resistant Drought Master cattle were infected with Babesia spp. but 43.7% of the Herefords were infected with Babesia spp. Although tick resistance appears to have an impact on preventing infection with Babesia spp., it was not effective in preventing infection with Theileria mutans as all cattle of both breeds were infected with this pathogen. Thus resistance to tick infestation provides protection from ixodid feeding as well as from some of the pathogens carried by the tick.

Host Cross Resistance to Tick Infestation

Trager (1939a) reported the acquired resistance to D. variabilis also provided cross resistant protection from challenge with Dermacentor andersoni. Since that time cross resistance has been reported by several other investigators. McTier (1981) divided D. andersoni resistant guinea pigs into groups and challenged them with D. andersoni, D. variabilis, Amblyomma americanum, or Ixodes scapularis. Considerable cross resistance was noted between Dermacentor species. Although the engorgement weights of the A. americanum and I. scapularis were significantly reduced, the number of ticks that engorged was not changed.

Whelen et al. (1984), using protein immunoblotting found sera from guinea pigs infested with adult D. andersoni recognized antigenic determinants in both D. andersoni larval extract and Amblyomma americanum ova extract; indicating shared antigenic determinants in different tick genera. Shared or core antigens could be very important in development of an immunization to artificially induce tick resistance (Wikel, 1982; Wikel et al., 1986). Some ixodid ticks apparently do not share common antigenic determinants, or at least not those which are associated with development or expression of acquired resistance. Wagland et al. (1985) found that cattle resistant to Haemaphysalis longicornis were as susceptible to Boophilus microplus as tick naive controls. Additionally, animals which were concurrently infested, developed resistance independently to each species based on exposure to that species.

### Tick Antigens

Purified and characterized tick antigens are needed to study host responses, to develop immunization regimens, and to study immunopathology in both host and parasite (Wikel and Whelen, 1986). Only a limited number of studies have attempted to isolate and characterize ixodid antigens (Willadsen, 1980; Wikel and Whelen, 1986; Wikel et al., 1986). In most cases this has involved identifying antigens that functioned in naturally acquired resistance and that are involved in hypersensitivity reactions at the bite site (Wikel et al., 1986).

Geczy et al. (1971) examined saliva from Boophilus microplus and found a macromolecule composed of three subunits which had esterase activity and degraded polysaccharide filtration medium. These esterases were skin tested in a tick sensitized animal and the reaction may have been due to increased vascular permeability in the skin. Prostaglandins was found in the saliva of Boophilus microplus and may be an important factor in the initiation and maintenance of the tick feeding lesion (Higgs et al., 1976; Dickinson et al., 1976).

Histochemical procedures allowed Schleger and Lincoln (1976) to demonstrate the presence of enzyme activity in skin of tick naive hosts immediately adjacent to larval Boophilus microplus mouth parts. Enzymes demonstrated in the dermis adjacent to larval mouth parts were: carboxylic ester hydrolase, triacylglycerol lipase, aminopeptidase, and monophenol monooxygenase. Two allergens have been separated and purified from unfed Boophilus microplus larvae (Willadsen et al., 1978). On

intradermal injection into previously infested cattle, these allergens elicited immediate hypersensitivity reactions and positive Prausnitz-Kustner reactions. Reactions were specific as these changes were not obtained with unexposed controls. This reaction was positively correlated to degree of resistance, or previous tick exposure (Willadsen et al., 1978).

Willadsen and Williams (1976) isolated and characterized one allergen with esterase activity (allergen I). This esterase shared properties with the one reported in dermis adjacent to larval mouth parts (Schleger and Lincoln, 1976) and may well be identical (Willadsen and McKenna, 1983b). Allergen II, a double headed trypsin-chymotrypsin inhibitor (Willadsen and Riding, 1979), induced a much stronger immediate allergic skin reaction than allergen I: however, skin reactions to both allergens correlated with resistance levels in cattle (Willadsen et al., 1978). This double headed proteolytic enzyme inhibitor was found only in larvae, but was similar and cross reactive with an enzyme found in tick eggs (Willadsen and Riding, 1979; 1980; Willadsen and McKenna, 1983a).

Evidence that these enzymes are injected into host tissues is the immediate hypersensitivity reaction obtained in resistant cattle, but not in unexposed animals, following intradermal injection of these substances (Willadsen and Riding, 1979). Willadsen and McKenna (1983b) showed that an esterase and a proteolytic enzyme inhibitor, from Boophilus microplus, aggregate and bind nonspecifically to other proteins via free sulphhydryl groups. Aggregation and nonspecific binding to other proteins result in tick proteins being retained in

host skin for longer periods than other proteins tested (Willadsen and McKenna, 1983b).

Binta et al. (1984a) isolated an allergen, from an extract of Rhipicephalus appendiculatus larvae, that when injected intradermally induced an immediate hypersensitivity reaction in tick resistant cattle but not tick naive cattle. When a standardized concentration of allergen was used the diameter of the reaction was found to correlate with the age and history of tick exposure. Presence of a positive passive cutaneous anaphylactic reaction where serum from a tick resistant steer was injected intradermally (ID) into a tick naive steer, but not where serum from a tick naive steer was injected, confirmed the presence of a homocytotropic antibody in tick resistance (Binta et al., 1984b). The swelling, caused by injecting allergen into resistant cattle, was reduced by pretreatment with mepyramine maleate, which inhibits histamine release, but was unaffected by phenylbutazone or acetylsalicylic acid, both of which are prostaglandins inhibitors (Binta et al., 1984c). Binta et al. (1984c) interpreted that these reactions showed the swelling was caused by histamine release from mast cells and that the swelling was not associated in any major way with prostaglandins.

More recently, use of gut antigens, which would induce a form of immunologic resistance that would act in the tick rather than in the host's skin has been suggested and investigated (Ackerman et al., 1980; Agbede and Kemp, 1986; Zorzopulos et al., 1973). Zorzopulos et al. (1978) isolated specific gut antigens in the form of subcellular fractions from Boophilus microplus which were high in both alkaline and

acid phosphatase activities. These enzymes are associated with absorptive surfaces and are markers for brush border fragments. The kinetics of these enzymes regarding pH and their association with the cell membranes were characterized (Zorzopulos et al., 1978). Although these enzymes were not used in immunization trials to determine their relationship to resistance, a subsequent study showed that cattle with naturally acquired resistance do produce antibodies to phosphomonoesterases. This antibody drops off in fourteen days presumably due to immunosuppression (Reich and Zorzopulos, 1980). Immunosuppression of this sort may result from suppression of T-helper cell activity by T-suppressor cells. Lymphocytes obtained from guinea pigs infested with D. andersoni showed antigen specific responsiveness from two days after a first infestation (day 7) until the fifth day (day 17) of a second infestation which then dropped to insignificant levels (Wikel et al., 1978). Reich and Zorzopulos (1980) found a maximum antienzyme activity at ten days which begins to decrease by 14 days which correlates with the time frame of antigen specific responsiveness reported by Wikel et al. (1978). Immunosuppression, specifically T-cell suppression, is supported by reduced in vitro responsiveness of lymphocytes to T-lymphocyte mitogens concanavalin A and phytohemagglutinin but not E. coli lipopolysaccharide, a B-lymphocyte activator, during tick infestation (Wikel, 1982b). The fact that cattle with naturally acquired resistance to tick infestation produce antibodies to phosphomonoesterases indicates that they are antigenic and that engorging ticks must introduce them into the host. Reich and Zorzopulos (1980)

suggested this was by regurgitation, but Tatchell (1967) indicated that phosphatases are found in tick saliva.

Immunization of cattle with crude extracts of adult female Boophilus microplus resulted in high levels of resistance involving gut damage to a large percentage of feeding ticks (Johnston et al., 1986). Agbede and Kemp (1986) suggest these findings are strong evidence that antigens important for this form of resistance are located on the luminal plasma membrane of digest cells in the gut caecae.

Working with Amblyomma americanum SGA and serum from resistant guinea pigs, Brown et al. (1984b) isolated a 20 Kdal SGA polypeptide which induced cutaneous anaphylaxis (Brown and Askenase, 1986) in tick sensitized animals. This antigen was isolated by precipitating radiolabelled SGA with serum from resistant guinea pigs. Resistance had been induced by either multiple infestations or by immunization with SGA and incomplete Freund's adjuvant (IFA) and was characterized by both tick rejection and reduced engorgement. The antigen was then electrophoresed by SDS PAGE, and autoradiography was performed to visualize it. Although several other bands could be seen, this 20 Kdal band was recognized only by serum from animals actively infested or immunized with SGA + IFA which manifested resistance by both reduced engorgement and tick rejection. Serum from guinea pigs which had been immunized with SGA + CFA did not recognize this antigen. The resistance manifested by these guinea pigs consisted only of decreased engorgement. Immunization with this 20 Kdal antigen induced resistance to infestation, but the level of resistance was not equal to that obtained by immunizing with crude SGA + IFA (Brown and Askenase, 1986).

Although the 20 Kdal protein appeared to have a significant role in induction of resistance, it did not induce the same level of resistance as crude SGA which may be a function of concentration injected. An alternative explanation may be that other proteins are involved in induction of resistance. Data from Brown and Askenase (1986) showing that fractions from the immunoaffinity column which did not contain the 20 Kdal polypeptide induced skin reactivity and a decrease in engorgement weight of ticks infesting guinea pigs immunized with these fractions seems to support this latter explanation.

Whelen et al. (1984) were the first to report on the identification of immunogenic components in extracts of larva and ova using immunoblotting. Dermacentor andersoni larval extract and Amblyomma americanum ova extract were fractionated into their polypeptide components using SDS PAGE and were then electroeluted to nitrocellulose. Serum from uninfested animals was used as a control while sera from guinea pigs infested four times with adult Dermacentor andersoni were used to identify immunogenic polypeptides. Sera from uninfested guinea pigs reacted with 41 and 65 Kdal bands in Dermacentor andersoni larval extract. This indicates that normal sera have antibodies specific for epitopes on these molecules, or cross reactivity with a common antigen. Sera from resistant animals recognized polypeptide components with molecular weights of approximately 25, 35, 41, 65, and 98 Kdal. These same sera showed cross reactivity by recognizing components with approximate molecular weights of 65, 93, 98, and 111 Kdal in the Amblyomma americanum ova extract. These studies show that sera from resistant animals recognize epitopes which must be common in

different life cycle stages of a given species as well as epitopes which are shared by ixodid ticks in different genera. Further studies showed that sera from rabbits infested with adult Amblyomma americanum, calves infested with adult Amblyomma americanum, and guinea pigs infested with adult Dermacentor andersoni all recognized antigens in Dermacentor andersoni larval extract indicating similar molecules may be introduced into host tissues during feeding by different genera of ixodids (Wikel and Whelan, 1986). Conserved epitopes may be significant candidate molecules for use in immunization (Wikel, 1982; Wikel et al., 1986).

Shapiro et al. (1986) also used immunoblotting with antibodies from resistant guinea pigs to detect immunogenic components in extracts of Rhipicephalus appendiculatus salivary gland, gut, and cement substance as well as larval and nymphal extracts. Most of the antigens detected were present in salivary gland. Many of these were also detected in cement substance. The presence of salivary antigens in this cement substance may have been due to salivary contamination which was not washed off the surface or may have been incorporated into the cement which is also produced in salivary gland acini and deposited in layers. Gut preparation contained several antigens with the same molecular weights that were recognized in salivary gland, including a 31 Kdal component which was present in a higher concentration in the gut preparation. Higher molecular weight components of saliva were not found in gut. The following is a list of the molecular weights of antigens from adult tick salivary gland recognized by this sera: 16, 20, 25, 28, 35, 46, 58, 77, 88, 94, and 120 Kdal. Instar specific

antigens may explain why resistance to one life cycle stage does not necessarily provide protection against another (Shapiro et al., 1986). It is apparent that many different immunogenic materials are inoculated into the host animal and that tick resistance is a complex phenomena probably elicited by multiple tick antigens (Shapiro et al., 1986).

Gill et al. (1986) using silver stained SDS PAGE gels found that protein bands in salivary gland extract from Hyalomma anatolicum anatolicum increased in number from 20 in unfed females to a maximum of 45 in females fed 96 hours. Nineteen protein bands were observed in the electrophoretic pattern of saliva collected from females fed for 96 hours. All of the saliva antigens and twelve of those from salivary gland extract were shown to be glycoproteins by staining with concanavalin A horseradish peroxidase. One band in saliva with a molecular weight of 96 Kdal (antigen III) made up the bulk of the salivary protein and showed nonspecific esterase and aminopeptidase activity. Antigen I, with a molecular weight of 130 Kdal was the next most prominent protein band in saliva and showed acid phosphatase activity. Antigen II had a molecular weight of 103 Kdal. These three antigens all induced immediate hypersensitivity reactions when injected intradermally into previously infested rabbits. Antigens II and III also induced strong delayed hypersensitivity reactions. Gill et al. (1986) stated that a wide range of proteins are involved in development of acquired resistance to infestation (Gill et al., 1986). Recognition of antigens by host sera confirms that they were antigenic upon injection into host tissues during engorgement.

Artificial Induction of Resistance

## Whole Tick Extracts

Trager (1939a), having recognized resistance was an immunological process, was the first to induce a degree of resistance to tick infestation in guinea pigs by intracutaneous inoculation of an extract of whole Derma-centor variabilis larvae. Since that time, other workers have had varying degrees of success using crude whole tick extracts of various life stages or tissues of different ixodid tick species in inducing resistance to infestation. Gregson (1941) reported inducing resistance to infestation by Derma-centor andersoni in guinea pigs with subcutaneous injections of emulsified, half engorged Derma-centor andersoni nymphs. Bagnall (1975) working with the guinea pig - Ixodes holocyclus system and using a larval extract, induced a high degree of resistance to infestation which was characterized by rejection of 38-68% of a challenge with 200 larvae. Multiple injections of the larval extract produced a more significant degree of resistance than a single injection. Ackerman et al. (1980) did not obtain a significant degree of resistance to infestation in rats injected with a whole tick extract of unfed adult Derma-centor variabilis. This was in contrast to Trager's (Trager, 1939a) experience with Derma-centor variabilis but may have been due to using unfed instead of partially engorged ticks or due to using a different host species. Allen and Humphreys (1979) found that antigens prepared from unfed female Derma-centor andersoni were

ineffective while those prepared from organs of ticks which had fed for five days induced resistance.

#### Organ Extracts

Trager (1939a) speculated that since only the ticks mouth parts contact host tissue, it was probable that some antigenic material in the saliva was injected into the host. Thus guinea pigs were immunized with crude extracts of salivary gland and digestive tract from Dermacentor variabilis (Trager, 1939b). The greatest degree of resistance was obtained using salivary gland extract (Trager, 1939b), but, unfortunately, statistical analysis of results was not presented. Immunization with salivary gland extracts induced resistance to challenge infestations with Hyalomma anitolicum excavatum (Kohler et al., 1967) and Rhipicephalus sanguineus (Garin and Grabarev, 1972). Brossard (1976) immunized calves with Boophilus microplus salivary gland extract prepared from partially engorged female ticks. This resulted in resistance which reduced the number of ticks able to engorge successfully. Wikel (1981) induced a high degree of resistance in guinea pigs using salivary gland antigens (SGA) from partially engorged, adult, female Dermacentor andersoni. Various routes of administration and use of two different adjuvants were evaluated. Intradermal administration of either one or ten micrograms of SGA induced resistance which was not significantly different statistically than that found in guinea pigs made resistant by repeated infestation (Wikel, 1981).

Brown et al. (1984) induced resistance to infestation by Amblyomma americanum larvae in guinea pigs by immunizing with 280 micrograms of SGA plus incomplete Freund's adjuvant (IFA) per animal via foot pad injections. This resistance was manifest by rejection and reduction in engorgement weight of larvae which was nearly equivalent to resistance acquired by active sensitization. Cement, which is produced in the salivary glands and acts as an attachment mechanism (Moorehouse, 1967), was found to give a level of protection similar to that obtained using SGA at twice the concentration (Brown et al., 1984b). In contrast, attachment cement from Dermacentor andersoni, washed prior to use, did not stimulate lymphocyte blastogenesis in lymph node cells from resistant guinea pigs nor did it induce skin reactions in resistant guinea pigs that differed from reactions in control animals (Wikel et al., 1978). The differences observed for Amblyomma americanum and Dermacentor andersoni attachment cement may indicate species specific differences in composition of attachment cement. Other possibilities are that since Brown et al. (1984b) collected attachment cement from tick mouth parts it may have been contaminated with other tick products, as it was not washed prior to use. Immunizing with cement may have exposed antigenic determinants, such as in the internum cement, that are not normally exposed to the host immune system through attachment and feeding.

Immunization with tick antigens other than those associated with the salivary gland have also been evaluated. Allen and Humphreys (1979) used two antigen preparations from partially engorged, adult, female Dermacentor andersoni. Antigen I, prepared from midgut and

reproductive organs, was evaluated in guinea pigs and calves. Antigen II, prepared from all internal tick organs, was only evaluated with guinea pigs. Ticks from guinea pigs immunized with antigen I at a rate of 1.2 mg/Kg of body weight, had reduced engorgement weights and produced few eggs from which no larvae hatched. The ticks collected from immunized calves had reduced engorgement weights, produced fewer eggs and fewer larvae than ticks from control calves. Ticks placed on guinea pigs immunized with antigen II neither engorged nor laid eggs.

Ackerman et al. (1980) removed the gut diverticula from adult male and female Dermacentor variabilis that had fed for 48 to 72 hours on rats. These gut diverticula were used to prepare soluble and particulate midgut extracts. Ticks, feeding on rats immunized subcutaneously with these midgut extracts, showed significantly reduced engorgement weights, egg production, and egg hatch; but did not experience increased fatality. Previous attempts to immunize with SGA, or whole tick extracts, had attempted to mimic naturally acquired resistance. This approach caused no reaction at attachment sites. Immunization with midgut antigens resulted in the production of antibodies which apparently did not function in host tissues at the bite site but instead acted on internal tick organs (Ackerman et al., 1980).

#### Antigen Prepared from Adult

#### Female Boophilus microplus

Immunizing cattle against Boophilus microplus (Johnston et al., 1986; Kemp et al., 1986; Agbede and Kemp, 1986) with an antigen,

apparently enriched for tick gut epitopes, resulted in a protective response which conforms to the concept presented by Ackerman et al. (1980). Cattle were immunized, over a seven week period, with a series of three immunizations composed of crude extract of Boophilus microplus adult female ticks diluted in either PBS or Freund's complete adjuvant (FCA) and were administered at various anatomical sites by either the subcutaneous or intramuscular route (Johnston et al., 1986).

Although the degree of resistance varied from animal to animal, with seven of fifteen showing good resistance, five of fifteen were intermediate, and the last three showed poor resistance, the resistance was still effective at fourteen weeks. Tick populations on these resistant animals were reduced by 70% compared to controls. Immunized cattle challenged twice with 20,000 larvae reduced the tick return by 93% compared to controls and tick weights were reduced by 95% (Johnston et al., 1986). The variation in resistance of immunized cattle may represent genetic variation in expression of immune response capabilities (Wikel, 1983; Whelen et al., 1984).

Many of the ticks, collected from immunized cattle, had gut damage (Johnston et al., 1986). Although antibody levels to the extract increased, there was no direct relationship between immunity and level of antibody. This was probably due to the potentially large number of antigens irrelevant to resistance in the extract (Johnston et al., 1986). It could also have been due to the impact of other immune mechanisms such as cell mediated immunity which are not directly related to antibody.

This immunization appears to have been stage specific, having had no effect on larvae while there were a progressive number of female tick deaths throughout feeding. Up to 60% of females had gut damage. Some of the adult males on immunized animals also suffered gut damage, but, none of the adult ticks on control animals showed any gut damage. The resistance reaction was very different than the normal active acquired resistance as no hypersensitivity reaction or exudate was seen at the site of tick attachment on any animals (Kemp et al., 1986).

Part of this rejection mechanism involved gut damage. In vitro feeding studies of ticks showed that complement must be present for gut damage to occur (Kemp et al., 1986). Gut damage was apparent because host erythrocytes were found in the hemolymph. Hematocrit values of up to 10% were obtained for hemolymph of ticks fed on immunized hosts. These high concentrations of host erythrocytes resulted in ticks which were an abnormal red color. (Johnston et al., 1986; Agbede and Kemp, 1986; Kemp et al., 1986). After gut damage occurred, host neutrophils and eosinophils escaped into the hemolymph and attacked muscles, malpighian tubules, and male accessory reproductive gland (Agbede and Kemp, 1986). Gut damage is apparently due to host response after exposure to these antigens in the extract. This response is a different response which could lead to greater levels of host resistance than is acquired by repeated tick infestations (Agbede and Kemp, 1986).

Research Methods

Multiple immunogenic molecules, which can be found in various tick extracts, have been shown to be involved in the complex mechanism of resistance to tick infestation (Whelen et al., 1984; Shapiro et al., 1986; Gill et al., 1986). The goal of this research was to separate immunogenic components of tick extracts and determine if they have a role in inducing resistance to tick infestation. A long range objective of research in this laboratory is to develop an immunization capable of artificially inducing resistance to tick infestation. Development of immunological and biochemical techniques with increased sensitivity and specificity have resulted in tools which can be used to analyze the significance of specific immunogens on the development of resistance to tick infestation. Specific tools used in this endeavor include: membrane ultrafiltration, gel filtration chromatography, electrophoretic analysis, immunoblotting, production of monoclonal antibodies, and Dot-ELISA.

Defined molecular weight range fractions of the tick extracts used in this study were prepared using membrane ultrafiltration. Blatt et al. (1965) demonstrated that ultrafiltration using membranes formed from the complex interaction product of polyanions and polycations (Diaplex or Diaflo membranes, Amicon, Blatt et al., 1965; 1967) concentrated protein solutions rapidly with no signs of denaturation. Membranes are available to fit specific ultrafiltration tasks and capable of screening solutes of varying molecular weights (Blatt et al., 1965). The chief advantage of this ultrafiltration technique is

that it rapidly and simply resolves complex mixtures into size graded classes, but one achieves limited fractionation rather than total resolution (Blatt et al., 1967).

Gel filtration chromatography using a Sephadex G-200 column was also used to fractionate antigens. Sephadex (Gel filtration, 1972) is a bead-formed gel prepared by cross-linking dextran with epichlorohydrin. Containing a large number of hydroxyl groups, the gel is very hydrophilic and swells readily in water and electrolyte solutions. The gel beads contain pores which function as the stationary phase. Very large molecules, above the exclusion limits, never enter the stationary phase, but move through the column with the liquid phase. Molecules which can enter the gel pores, spend time in the stationary phase, and move more slowly through the column. Thus, molecules are eluted in order of decreasing molecular size. This is basically a sieving action. Sephadex G-200 has a molecular weight fractionation range of five to 800 Kdal for polypeptides and globular proteins. In addition to fractionation of solutes, these columns can be calibrated and used to determine molecular weights of proteins (Ackers, 1964; Gel filtration, 1972; 1984). By pooling specific aliquots, molecular weight range fractions of tick extracts similar to those obtained by ultrafiltration were prepared.

Fraction separations obtained by ultrafiltration and gel filtration chromatography were analyzed by SDS-PAGE gels stained with the Morrissey modified silver stain (Morrissey, 1981). This staining method is 100 times more sensitive than Coomassie blue staining (Morrissey, 1981). Polyacrylamide gels are the medium of choice for

zone electrophoresis of most proteins because it can be reproducibly prepared with a wide range of pore sizes (Hames, 1981). The principle of polyacrylamide electrophoresis is that molecules are separated based on molecular charge and size. The sieving action of polyacrylamide results in high resolution (Hames, 1981). Superior resolution of complex protein mixtures is obtained using the Laemmli SDS-discontinuous buffer system (Hames, 1981; Laemmli and Favre, 1973). In this system, the protein mixture is denatured at 100 C in the presence of excess SDS and a thiol reagent which cleaves disulphide bonds generating polypeptide subunits.. This results in most polypeptides binding SDS in a constant ratio of 1.4 g SDS/g of polypeptide. The very high negative charge of SDS negates the normal charge of the polypeptide and separation is then strictly according to polypeptide size. In addition to determining composition, molecular weight can be established by comparing the relative mobility to that of molecular weight standard markers on the same gel (Hames, 1981). This method was used to determine the composition and molecular weight of components and/or subunits of tick extracts.

Involvement of multiple immunogenic molecules has been demonstrated by immunoblotting. Immunoblotting is an advanced immunological procedure developed by Towbin et al. (1979) in which complex mixtures of proteins are first fractionated into their polypeptide components by polyacrylamide gel electrophoresis, usually in the presence of SDS, and are then electroeluted to a nitrocellulose sheet. The polypeptide bands are immobilized on the nitrocellulose in exactly the same pattern found in the gel. This nitrocellulose sheet can then be

stained for protein pattern or reacted with antibodies (first antibody) after blocking unoccupied protein binding sites. Visualization or immunodetection of bands is accomplished by reacting the nitrocellulose with a second antibody (antispecies immunoglobulin) which has been tagged with radioisotopes, fluorochromes, or one of the very sensitive enzyme tags such as horseradish peroxidase and visualized by autoradiography, illumination with ultraviolet light, or addition of a precipitable substrate respectively. Use of horseradish peroxidase allows detection of as little as 100 pg protein. This was an important advance in that smaller amounts of antibody, or ligand, can be used to determine reactivity and the problem of gel pore size limiting diffusion is avoided (Towbin et al., 1979; Gershoni and Palade, 1983). Although a faithful replica of SDS-PAGE gel patterns could be transferred, they were not quantitative (Towbin et al., 1979). The modifications of Burnette (1981) resulted in quantitative transfer of SDS-PAGE gel patterns to nitrocellulose. Greater than 90% of all proteins with molecular weights up to 100 Kdal were transferred in 20 hours. The sensitivity of immunodetection is a function of specific antibody titer; but Burnette (1981) was able to detect as little as one to two nanograms of protein using this system.

In a continuing effort to accurately define and acquire antigens, which are involved in resistance to tick infestation, monoclonal antibodies are being produced to immunogenic tick polypeptides. These monoclonal antibodies will eventually be used in immunoaffinity techniques to obtain purified tick antigens. Cell fusion techniques were used to develop monoclonal antibody producing hybridoma cell

lines. Kohler and Milstein (1975) introduced cell fusion techniques utilizing Sendai viruses to fuse mouse myeloma cells with syngeneic antibody producing spleen cells for the production of monoclonal antibodies. These cells produced and secreted the products of both parental strains (Kohler and Milstein, 1975). Use of a mutant myeloma cell line such as NS1/1-Ag4-1 (abbreviated NS-1), which is a nonsecreting variant not expressing heavy chain, results in only the spleen cell parent's antibody being expressed (Kohler and Milstein, 1976). Gefter et al. (1977) simplified performing fusions by replacing the Sendai virus with polyethylene glycol (PEG) which increased the hybridization frequency by 300 fold. This technique has allowed the isolation of hybridoma cell lines which secrete monoclonal antibodies of any desired specificity (Bruck et al., 1986). This technique has progressed to the point that it is now common to produce monoclonal antibodies to distinct epitopes on a single polypeptide. These can then be used to topographically map the polypeptide (Freeman et al., 1985).

Injection of hybridoma cells into a syngeneic host results in solid tumors and production of large quantities of these monoclonal antibodies (Kohler and Milstein, 1975; 1976). Production of ascites fluid, by injecting hybridoma cells into the peritoneal cavity of mice, is a routine procedure to obtain large quantities (10-100 mg/mouse) of high titer monoclonal antibody. Antibody of much higher titer is obtained from ascites than can be obtained from culture supernatant (Bruck et al., 1986; Campbell, 1984; Goding, 1983).

Hawkes et al. (1982) introduced a procedure they called a dot immunobinding assay which also used nitrocellulose as a solid matrix for binding antigen to be used in detection of antibody. The antigen is dotted directly onto nitrocellulose rather than blotted from a gel onto nitrocellulose which is a significant simplification. Used in an enzyme linked immunosorbent assay (ELISA), nitrocellulose disks have two significant advantages over coated microtiter plate wells: Use much less antigen, as little as 0.1 microliter produces dots which are 0.3 mm in diameter and are still clearly visible; and does not require instrumentation to interpret as the disks provide a white background against which the blue dots are very easily seen (Hawkes et al., 1982). Pappas et al. (1983) adapted the procedure of Hawkes et al. (1982) for a diagnostic test for visceral leishmaniasis by dotting whole Leishmania donovani promastigotes, as antigen, on the nitrocellulose disks. At a diagnostic titer of 1:32 98% of true positives were positive, while the procedure had a false positive rate of only two percent. The procedure was very sensitive, being able to detect as little as 0.1 ng of human antibody. Reproducibility was excellent with 90% of the samples showing no variation in end-point titration, while the other ten percent varied by only plus or minus one dilution (Pappas et al., 1983). Antigen disks were shown to be very stable. Disks stored at -20 C were stable over 270 days; while disks maintained at ambient temperature had no significant loss of reactivity in 30 days (Pappas et al., 1984). Whelen et al. (1986), adapted the procedure as used by Pappas et al. (1983) to study development of antibody response of guinea pigs infested with Dermacentor andersoni to Dermacentor

andersoni SGA. Both immunoblotting and Dot-ELISA techniques have been used in this study.

## MATERIALS AND METHODS

### Ixodid Ticks

Two species of ixodid ticks, Amblyomma americanum and Dermacentor andersoni, were used in this research. Ticks were maintained in 1.5 dram colorless, glass vials, stoppered with cloth wrapped cotton plugs. These vials were kept at room temperature in a dessicator over a saturated solution of potassium nitrate to maintain a relative humidity of  $85 \pm 5\%$ . Ticks were originally obtained from the United States Livestock Insects Laboratory, USDA, ARS, Kerrville, Texas pathogen free colony which was established in 1956 from ticks collected from cattle in Bexar County, Texas. The tick colony has been maintained pathogen free for eight years in this laboratory.

Tick propagation, for colony maintainance, was performed either on the ears of white male Hartley guinea pigs or the backs of New Zealand White rabbits. Ticks were confined to guinea pig ears by the top 25 millimeters (mm) of amber seven dram lock top vials (Kerr Glass Manufacturing Corp., Lancaster, PA) which were secured by cloth tape. Additionally a plexiglass collar prevented the vial from being removed by grooming. Rabbits were shaved circumferentially between the fore and hind legs. The top 50 mm of a Nalgene wide-mouth polyethylene bottle (Nalge Company, Rochester, NY) with a 43 mm closure was fastened to the rabbits back by cloth tape and contact cement (Red Devil Inc., Union NJ). These harnesses confined the ticks to a limited area and protected them from grooming activities.

Laboratory Animals

Hartley male guinea pigs weighing approximately 350-400 grams (g) were purchased from Bio-Labs Corp, St Paul, Minnesota. New Zealand White rabbits, weighing approximately 1.5-2.0 kilograms (Kg), were obtained from the University of North Dakota Animals Resources Facility breeding colony. White BALB/c mice used for monoclonal antibody production were obtained from Harlan Sprague Danley, Inc., Madison, Wisconsin at two to three weeks of age. All animals were fed the appropriate pellet diet (Ralston Purina Company, Richmond, IN) and water ad libitum. Rabbits and guinea pigs were housed individually in stainless steel mesh cages, while mice were maintained in groups of two to five in clear plastic cages with stainless steel wire tops. The temperature was maintained at 22 degrees Centigrade (C) and the photoperiod was 12 hours.

Blood Collection and Processing

Blood was collected from guinea pigs by cardiac puncture using 1 1/2 inch, 20 guage needles and ten milliliter (ml) syringes. The blood was placed in sterile glass tubes, allowed to coagulate at room temperature for two hours, rimmed with a wooden applicator stick and then placed in a 4 C refrigerator overnight. The tubes were centrifuged at 200 x g in a refrigerated centrifuge (International Equipment Co., Needham Hts., MA) and serum removed with sterile Pasteur pipets.

Serum was aliquoted into five milliliter screw top glass vials, frozen and maintained at -20 C.

Small amounts of blood were taken from rabbits by nicking the marginal ear vein and collecting the blood in heparinized capillary tubes (Sherwood Medical industries, ST. Louis, MO). The capillary tubes were spun in a hematocrit centrifuge (International Equipment Company, Boston, MA) to separate the plasma. Larger volumes of blood were taken, using 18 gauge 1 1/2 inch needles and 20 ml syringes, by blind cardiac puncture or the animal was anesthetized and exanguinated by open chest cardiac puncture. Blood was processed in the same manner described for guinea pig blood.

#### Antigen Preparation

Tick extracts used in this investigation were prepared from both Amblyomma americanum and Dermacentor andersoni ova and salivary glands. Amblyomma americanum gut extract, brush border fragments, and 27000 x g supernatant were also prepared and used in immunization regimens.

Ticks were dissected to obtain the desired organs for extract preparation. This was accomplished by embedding adult ticks in a paraffin filled petri plate such that the extended legs, head, and ventral side were secured in the paraffin leaving the dorsal side exposed. The dish was then flooded with 0.15 M phosphate buffered saline pH 7.2 (PBS, Appendix II) and placed on the stage of a dissecting microscope. Using a Bard-Parker # 11 disposable scalpel blade (Becton Dickinson and Co., Rutherford, NJ) the scutum and exoskeleton

were cut off and removed with tweezers. Gut caecae or mesenterons were normally removed first and placed in a 1.5 ml conical centrifuge tube (Centaur Sciences, Inc., Stanford, CT) with 100 microliters of PBS for extract or component preparation. The internal cavity was then cleaned by aspiration with a Pasteur pipet. The white grape-like clusters making up the salivary glands were collected, avoiding tracheal elements, and placed into a 1.5 ml conical plastic tube with 50 microliters of PBS to prepare salivary gland antigen (SGA). All tubes containing organs were kept on wet ice.

#### Salivary Gland and Gut Extract

Extracts of salivary gland and gut were prepared by grinding the organs in the conical tubes containing PBS with a glass rod. The resulting emulsion was centrifuged at 13,000 x g for 20 seconds in a Microcentrifuge (Fisher Scientific Co., Pittsburgh, PA). This was followed by three cycles each composed of three steps: (1) sonication at 55,000 cycles per second for 15 seconds in a Bransonic 12 (Branson Cleaning Equipment Co., Shelton, CT), (2) cooling on ice, and (3) centrifugation at 13,000 x g for 20 seconds, except for the last centrifugation which was for five minutes. The supernatant was removed, placed into appropriately labelled tubes and kept frozen at -20 C.

## Ova Extracts

Ova extract was prepared by pooling the ova produced by several females. The ova were dispersed with acetone in a funnel with filter paper and surface sterilized by soaking in White's solution (Appendix II). White's solution was removed by washing with copious amounts of distilled water. The ova were then ground in a tissue grinder with 1.5 ml of PBS. This emulsion was sonicated at 55,000 cycles per second for 40 seconds, then cooled in an ice bath. After three cycles of grinding, sonicating and cooling, the suspension was pipetted into two 1.5 ml conical tubes. These tubes were then centrifuged for five minutes at 13,000 x g. The supernatant was aliquoted into tubes, labelled, and frozen at -20 C until ova extract was needed.

Amblyomma americanum Gut Antigens

Fifty newly molted adult ticks were dissected as described above. The gut diverticula were collected in a 1.5 ml conical tube containing 100 microliters of PBS. This tube and its contents were subjected to ten cycles of sonication for 30 seconds at 55,000 cycles per second followed by cooling in an ice bath for one minute. No centrifugation was performed and the emulsion of fine particles was frozen at -20 C until used. This preparation was sonicated for 15 seconds prior to use to evenly distribute the fragments.

Brush Border Fragments and  
27,000 x g Supernatant

Brush border fragments were prepared from mesenterons following the method utilized by Houk et al. (1986). Fifty unfed Amblyomma americanum adult ticks were dissected with the gut diverticula placed into a 1.5 ml conical tube containing 0.5 ml of isolation buffer (Appendix II) and held on ice. Gut diverticula were disrupted in a tight fitting ground glass tissue grinder. The resulting suspension was adjusted to a concentration of 10 mM CaCl<sub>2</sub> and incubated for 15 minutes on ice. The suspension was clarified by centrifugation at 6,000 x g for 15 minutes in a Sorvall RC2-B refrigerated centrifuge (Dupont Company, Willmington, DE). The supernatant was then centrifuged at 27,000 x g for 30 minutes. The supernatant was labelled 27,000 x g supernatant and the pellet, resuspended in 50 microliters of PBS, was labelled brush border fragments. These were frozen at -20 C.

All tick antigens were characterized by protein content and polypeptide composition. Protein concentration was determined by Bradford dye-binding protein assay (Bio-Rad Laboratories, Richmond, CA). Polypeptide composition was determined by SDS-PAGE performed under reducing conditions (Laemmli, 1970). These gels were stained with a very sensitive silver stain (Morrissey, 1981).

Electron Microscopy

To observe salivary gland acini and gut brush border, microvilli, a portion of Amblyomma americanum salivary gland and gut were fixed in Karnovsky's fixative (Appendix II) at 4 C overnight. These tick tissues, were rinsed three times in 0.2 M, pH 7.2, cacodylate buffer (Appendix II), then post fixed with 1% osmic acid at 4 C, in the dark, for 90 minutes. Following three rinses in distilled water the samples were dehydrated using the following series of solutions, each with an increasing concentration of ethanol: five minutes each in 50%, 70%, 80%, and 95% ethanol. This was followed by ten minutes in 95% and ten minutes in 100% ethanol. The final dehydration was in two 100% ethanol washes for 20 minutes and 30 minutes respectively. Three washes in propylene oxide for 20 minutes each, were followed by two increasing concentrations of EMBED-812 (Polyscience, Fort Washington, PA) in propylene oxide to pure EMBED-812 for one hour each. The specimen was then embedded in fresh EMBED-812 and cured first at 37 C for 24 hours and then at 60 C for 48 hours. The embedded specimen was thin sectioned (600 angstroms thick) using a diamond knife on a Sorval MT2-B Ultramicrotome (Dupont Instrument, Newton, CT). The sections were placed on grids and then stained in 2% uranyl acetate (Appendix II) for five minutes. Then the grids were rinsed in distilled water, followed by staining in 0.018% lead citrate (Appendix II) for five minutes. The final step in preparation was a thorough rinse in distilled water.

Sections were placed on a grid, observed and photographed using a

model JEM-100S Transmission Electron Microscope (JEOL Ltd., Tokyo, Japan) which had magnification capability from 100x - 200,000x.

Preparing Fractions of Ova Extracts  
with Defined Molecular Weight Ranges

Ultrafiltration

Both Dermacentor andersoni and Amblyomma americanum ova extracts were fractionated into defined molecular weight range components. Molecular weight ranges which were prepared and used, in addition to whole ova extract, were 100 Kdal and greater, 50 - 100 Kdal, 30 - 100 Kdal, and 10 -30 Kdal fractions. Fractionation of whole ova extract was accomplished by use of a series of Diaflo Ultrafilters (Amicon Corporation, Danvers, MA) consisting of YM-100, XM-50, YM-30, and YM-10, in a model 8010 Ultrafiltration Cell (Amicon Corporation, Danvers, MA). The molecular weight cut off values for the filters were > 100 Kdal, > 50 Kdal, > 30 Kdal, and > 10 Kdal respectively.

The whole ova extract was diluted in ten milliliters of PBS and filtered through the YM-100 filter using ten pounds per square inch (psi) of nitrogen pressure. The retentate was concentrated to approximately one milliliter, labelled 100 Kdal and greater, and frozen at -20 C. The filtrate having passed through the YM-100 filter was collected and filtered either through the XM-50 filter or the YM-30

filter to produce the 50 - 100 Kdal or 30 -100 Kdal fraction respectively in the retentate. These were appropriately labelled and frozen at -20 C. The filtrate was filtered through the YM-10 filter with the resulting retentate labelled 10 -30 Kdal and frozen, while the final filtrate was discarded. Protein concentration was determined for the fractions by Bradford dye-binding assay (Bio-Rad Laboratories, Richmond, CA) and SDS-PAGE, as described below, were performed to characterize the fractions and confirm purity.

#### Gel Filtration Chromatography

A second method of preparing defined molecular weight range fractions of Amblyomma americanum ova extract was column gel filtration chromatography. The column used was a 2.6 cm x 82 cm Sephadex G-200 column, with a 435 ml bed volume. This column was run at 4 C with a flow rate of 15.6 ml / hour. Eight milliliter fractions were collected and screened by a Beckman DB ultraviolet (UV) spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) at 280 nanometers (nm) for protein content. Fractions making up protein peaks were pooled and concentrated using membrane ultrafiltration. The sample applied was six milliliters of Amblyomma americanum ova extract with a total protein content of 76.6 milligrams (mg).

Bradford Dye-binding Assay for Protein

The reagents for the Bradford dye-binding assay for protein were purchased from Bio-Rad (Bio-Rad Laboratories, Richmond, CA). Concentrated dye reagent was diluted (1:5) one volume of dye reagent with four volumes of distilled water and filtered through Whatman #1 filter paper. Five milliliters of this filtered dye solution was mixed with each sample on a vortex mixer and then incubated at 22 C for 20 minutes. At the end of incubation each assay tube was read spectrophotometrically at 595 nm in a Spectronic 20 (Scientific Products, Minneapolis, MN). A standard curve was drawn (O.D. vs protein concentration of standards) for samples having given concentrations of bovine serum albumin, which were assayed simultaneously with unknown samples. The value for the unknown sample was determined from the standard curve and calculations adjusting for dilution factors were performed.

Sodium Dodecyl Sulfate-PolyacrylamideGel Electrophoresis (SDS-PAGE)

SDS-PAGE, performed by the method of Laemmli (1970), was used to fractionate tick antigens in order to characterize them by the number and molecular weight of polypeptide components and subunits. This was also the first step in the immunoblotting procedure which will be described later.

First the glass plate sandwich was assembled and placed into the leveled casting stand. A 12% separating gel (Appendix II) was mixed and poured into the sandwich making a 1.5 mm thick 14 x 14 cm gel. This was immediately overlaid with distilled water and allowed to sit for one hour. The water was poured off and replaced with fresh distilled water. Then the unit was covered with parafilm and the gel allowed to finish polymerizing overnight. The next morning a 4% stacking gel (Appendix II) was mixed and poured on top of the separating gel after the water overlay was poured off. A channel comb was placed in the stacking gel immediately after it was poured and the gel was allowed to polymerize for one hour.

While the stacking gel was polymerizing, samples were diluted 1:4 with sample buffer (Appendix II), which contains an excess of sodium dodecyl sulfate and 2-mercaptoethanol, a thiol, and heated for four minutes at 95 C. The sample of SDS molecular weight markers contained: ovalbumin (45 Kdal), bovine serum albumin (66 Kdal), phosphorase B (92.5 Kdal), beta galactosidase (116.25 Kdal), and myosin (200 Kdal), (Sigma Chemical Co., St. Louis, MO) and was processed the same as all other samples. The channel comb was removed and the samples were each pipetted into a separate channel. The top chamber was attached to the glass sandwich and then filled with upper chamber buffer (Appendix II). The upper chamber attached to the glass sandwich was then inserted into the lower chamber of the Bio-Rad Protean Dual Vertical Slab Gel Electrophoresis Cell (Bio-Rad Laboratories, Richmond, CA) which contained electrode buffer (Appendix II). The lid was placed on the electrophoresis cell and was connected to the model 500/200 power

supply (Bio-Rad Laboratories, Richmond, CA). The power was set at 20 milliamperes (ma) until samples reached the interface of the stacking and separating gels. It was then turned up to 30 ma until the running dye front was within approximately 0.25 inch of the bottom of the gel at which time the power was turned off and the gel removed. Stacking gel was removed and discarded. Separating gel was either stained for protein or was cut so that the molecular weight markers and representative samples were stained for protein while the rest of the gel with duplicate samples was electroeluted to nitrocellulose as the first step in immunoblotting.

#### Protein Staining of SDS-PAGE Gels

Some of the early gels were stained with buffalo black (Allied Chemical Corp., New York, NY) and then placed in decolorizer (Appendix II) until the background was clear. The majority of gels were stained with the more sensitive silver stain.

Sensitive and reproducible, the Morrissey silver stain technique was used to detect polypeptides. Gel was removed from the electrophoresis apparatus and placed in a glass dish with 200 ml of 50% methanol, 10% glacial acetic acid to prefix for 30 minutes. This and all other incubations in this procedure were performed on a rotary platform mixer (American Hospital Supply Co., Miami, FL) at room temperature. The prefix solution was decanted and replaced with 100 ml of 2.5% gluteraldehyde and incubated for 30 minutes to fix the gel. The gel was then either soaked in distilled water overnight followed by

a 30 minute rinse in fresh distilled water, or rinsed in distilled water for two hours with the water being changed every 20 minutes. The gel was then reduced for 30 minutes with 50 microliters of dithiothreitol in 200 ml of distilled water. This solution was poured off without rinsing. Then the gel was stained for 30 minutes with 65 ml of 0.1% silver nitrate solution, which covered the gel. The gel was rapidly rinsed with distilled water, followed by one quick rinse with 50 ml of developer. Then 100 ml of developer was poured into the pan, which was sitting on a white sheet of paper on the rotary mixer. The developing gel was watched very closely until distinct bands were observed. At this point development was stopped by addition of five milliliters of 2.4 M citric acid (Appendix II) to the developer and gel. The gel was allowed to incubate for ten minutes with the citric acid. Gels continued to become darker for a while even after addition of citric acid. Then the gel was rinsed several times with distilled water. The gels were then placed in 0.03%  $\text{Na}_2\text{CO}_3$  plus ten milliliters of glycerol. The gels were either stored this way or sealed in uncoated cellophane sheets (Flexel, Covington, IN) and allowed to dry overnight at room temperature.

#### Immunoblotting

#### Electroelution

The first step of this procedure was to transblot or electroelute the fractionated antigen from the polyacrylamide gel to nitrocellulose.

This was done by using a Transblot Cell (Bio-Rad Laboratories, Richmond, CA) which functions like any other electrophoresis chamber, but has its electrodes oriented in the front and back of the chamber so that movement will be horizontal rather than vertical. Gel was taken from the SDS-PAGE procedure and equilibrated in transfer buffer (Appendix II) as were the nitrocellulose sheet, sponges, and filter paper pads. Gel holder was opened and a sponge and filter pad were placed on one side. Gel was placed on top of the equilibrated filter pad and the nitrocellulose was placed on top of the gel. Nitrocellulose must not be moved or repositioned. All bubbles were removed and good contact between the gel and nitrocellulose was assured. The remaining soaked filter pad and sponge were added. Gel holder was closed, locked, and placed into the Transblot Cell half filled with transfer buffer. The tank was then filled with transfer buffer (Appendix II). Power was set to 150 ma and the unit was in operation over night.

#### Immunolocalization

The next morning the nitrocellulose was removed. It was either dried and stored for use at a later time or used immediately for immunolocalization and immunodetection. Immunolocalization began by immersing the nitrocellulose in a solution of Tris saline pH 7.4 with 5% Blotto (Appendix II) for 30 minutes which blocks all unused protein binding sites on the nitrocellulose. This solution was discarded and the nitrocellulose was covered with Tris saline with 5% Blotto plus the

first antibody. First antibody was usually serum from a resistant or immunized animal or antibody of hybridoma origin. The nitrocellulose was incubated with the first antibody solution for 60 minutes at room temperature on a platform rotary mixer. This first antibody solution was saved and reused. The nitrocellulose was then washed three times with Tris saline with 5% Blotto and 0.05% Tween 20 (Appendix II). These washes complete immunolocalization in which first antibody will bind to electroeluted antigen epitopes it recognizes.

#### Immunodetection

Immunodetection or visualization involved incubating the nitrocellulose, after immunolocalization, with a second antibody. The second antibody was antispecies IgG to the first antibody was tagged, in this case with the enzyme horseradish peroxidase (HRPO). The second antibody was diluted in Tris saline plus 5% Blotto and then incubated with the nitrocellulose at room temperature for 60 minutes. Three washes in Tris saline plus 5% Blotto were followed by a five minute wash in Tris saline only. Addition of a precipitable substrate (Appendix II), (4-chloro-1-naphthal), and incubation for up to 30 minutes, resulted in blue colored bands where first antibody had recognized epitopes on polypeptides. Before the background color on the nitrocellulose got too dark, substrate was decanted and the nitrocellulose was washed three times with Tris saline. The nitrocellulose was dried for five to ten minutes at 56 C.

Skin Testing

Defined molecular weight range fractions and whole ova extract antigens were skin tested in guinea pigs which had been previously infested; one with Dermacentor andersoni larvae and one with Dermacentor andersoni nymphs. A tick naive guinea pig was tested as an unexposed control. The day prior to skin testing the entire ventral side on each of the guinea pigs was shaved. Each guinea pig was skin tested with seven antigens and PBS. The seven antigens were: (1) Dermacentor andersoni whole ova extract, (2) 100 Kdal and above fraction of Dermacentor andersoni ova extract, (3) 30 - 100 Kdal fraction of Dermacentor andersoni ova extract, (4) 100 Kdal and above fraction of Dermacentor andersoni ova extract, (5) 50 - 100 Kdal fraction of Dermacentor andersoni ova extract, (6) Dermacentor albipictus whole ova extract, and (7) Amblyomma americanum whole ova extract. Skin fold thickness and reaction diameters were measured using a caliper and recorded for each preselected skin test site. Then 20 micrograms of antigen, in a total volume of 100 microliters, was injected intradermally, with a 1/2 inch, 26 guage needle, at the preselected site for each antigen and the PBS control.

The injection sites were checked and observations recorded at 0.5, 1, 6, 24, and 48 hours for erythema, induration, diameter of reaction, and skin fold thickness. After the 48 hour readings, the animals were euthanized using CO<sub>2</sub>. A 3x3 mm biopsy was taken from each reaction site.

Biopsy Processing

Biopsy specimens taken at skin test sites and punch biopsies taken at tick attachment sites on infested guinea pigs were immediately placed in prelabelled glass vials containing Karnovsky's fixative held on ice. They were then refrigerated overnight at 4 C. Specimens were then given three, ten minute washes with PBS. This was followed by dehydration in increasing concentrations of ethanol as follows: (1) 50% ethanol for ten minutes, (2) 75% ethanol for 30 minutes, (3) four, 30 minute, washes in 95% ethanol, and (4) two, 30 minute, washes with 100% ethanol. The 100% ethanol was followed by two washes at 15 minute intervals with xylene. All the xylene was discarded except for enough to just cover the specimen, and melted paraffin (Peel-A-Way Scientific, Detroit, MI) was added. These vials were incubated at 60 C for 1 to 6 hours. The xylene wax mixture was quickly dumped leaving the specimen in the vial. The vial was filled with pure paraffin and incubated at 60 C for 18 to 24 hours. The specimen was then placed into a mold (Lipshaw, Detroit, MI) filled with paraffin, positioned, and allowed to harden overnight. These paraffin blocks were then mounted on wooden blocks (Lipshaw, Detroit, MI) and the face trimmed down for sectioning. Sectioning was performed using a rotary microtome (Leitz, West Germany) to cut sections which were seven micrometers thick. Sections were stretched on warmed water and adhered to acid cleaned slides using either egg white or gelatin.

### Tissue Staining

All sections were stained with Wolbach's (1919) modification of the giemsa stain to enhance detection of basophils. First the slides were rehydrated by placing them in a series of eight Coplin jars for five to ten minutes each. The first two jars contained xylene, the next two contained 100% ethanol, followed by one each of 95%, 70%, and 50% ethanol, and one containing distilled water. The rehydrated slides were placed in the giemsa stain (Appendix II) for 24 hours. The stain was rinsed off by very slowly running tap water into the Coplin jar for five minutes. Slides were individually differentiated with colophonium in ethanol (Appendix II). This reaction was stopped in 100% ethanol which begins the dehydration stage. Dehydration consists of two changes of 100% ethanol, approximately five minutes per change, followed by two changes of xylene. The slide was then coverslipped using Preservaslide (Matheson Coleman and Bell, Norwood, OH).

### Immunizations

#### Guinea Pigs

Four different immunization protocols, which incorporated various Amblyomma americanum antigens, were utilized to induce resistance to Amblyomma americanum infestation in white male Hartley guinea pigs. The efficacy of the immunizations were evaluated by challenging infestations of either Amblyomma americanum larvae or Amblyomma

americanum adults, and assessing engorgement weights, viability, molting to the next life cycle stage, ova production, and estimating hatch success.

Immunization protocol I evaluated the protection provided by defined molecular weight range fractions of Amblyomma americanum ova extract obtained through ultrafiltration. Two guinea pigs were immunized with Amblyomma americanum whole ova extract at a dosage rate of ten micrograms per 250 grams body weight. Two guinea pigs were immunized with 7.5 micrograms of a 100 Kdal and above fraction per 250 grams body weight. A third pair of guinea pigs were immunized with five micrograms of a 30 -100 Kdal fraction per 250 grams body weight. Initial immunizations incorporated an equal volume of incomplete Freund's adjuvant (IFA) with the antigen, while the remaining three immunizations, given on days 14, 21, and 28 were given without adjuvant. All immunizations were given subcutaneously (SC) in the nuchal region. In addition to the immunized animals, two guinea pigs, the two exposure controls, received two challenge infestations of 100 Amblyomma americanum larvae; and two guinea pigs, the one exposure controls, were challenged with 100 Amblyomma americanum larvae. On day 35 all immunized animals and the one exposure controls were challenged for the first time. The two exposure controls received their second challenge on day 35, with a seven day tick free period having been provided between the first and second infestations.

Immunization protocol II utilized fractions of Amblyomma americanum ova extract obtained by fractionation of whole ova extract on a Sephadex G-200 column. Pairs of guinea pigs were immunized with

either whole ova extract, peak 1, peak 2, or peak 3 fractions at a dose of 50 micrograms per 400 grams body weight. On day 0 and 14 the appropriate antigen was mixed with an equal volume of IFA and injected intramuscularly (IM). Immunizations given on days 21, 28, and 35 contained only antigen and were given by the SC route. The pair of guinea pigs functioning as PBS controls had PBS substituted for antigen, but were immunized on the same schedule and in the same way as the test guinea pigs. These animals as well as the one and two exposure controls were all challenged with 100 Amblyomma americanum larvae on day 42. The two exposure controls had a seven day tick free period between their first and second infestations.

Immunization protocol III added gut derived material to the antigens utilized in protocol II. Thus pairs of guinea pigs were immunized with 20 micrograms of antigen, whole ova, peak 1, peak 2, or peak 3, plus 20 micrograms of Amblyomma americanum gut antigen containing membrane fragments per 250 grams body weight. All animals were immunized IM on days 0 and 14 with the appropriate antigens in an equal volume of complete Freund's adjuvant (CFA). On days 29 and 36 antigens without adjuvant were given SC in the nuchal area. One and two exposure controls were managed in the same manner already described in protocol I and II except that the challenge which was given on day 42 consisted of four pair of adult Amblyomma americanum ticks, with two pair applied to each ear.

Immunization protocol IV further evaluated the effect of immunizing with gut antigens, more specifically with brush border fragments (BBF) and 27,000 x g supernatant obtained preparing BBF. Pairs of

guinea pigs were immunized intraperitoneally (IP) with either 1 microgram BBF per 250 grams body weight or with ten micrograms of 27,000 x g supernatant per 250 grams body weight. One control guinea pig was immunized IP with PBS. All animals were immunized with the appropriate antigens on days 0, 14, 21, and 28; and challenged with two pair of adult Amblyomma americanum per ear on day 35.

#### Mice

BALE/c, white, male mice were immunized in order to stimulate clonal proliferation of large numbers of cells of B-lymphocyte lineage which produced specific antibodies against tick antigens. These B lymphocytes, which produced antibodies against various epitopes on the immunogen, were mainly located in peripheral lymphoid tissues such as the spleen and lymph nodes. Thus, a spleen provided an enriched source of the desired cells of B-lymphocyte lineage for production of hybridoma cells.

In mouse immunization regimen I, five mice were immunized with Dermacentor andersoni ova extract. The initial immunization contained 100 micrograms of antigen in CFA. One half of this was given IM in the hip region, and one half was given SC in the nuchal area. Booster immunizations of 50 micrograms were given IP at 14 days, 21 days, and three days prior to performing the fusion.

In immunization regimen II, mice were immunized naturally by active infestation. Natural immunization was performed by infesting four mice with six Dermacentor andersoni nymphs per mouse. Nymphs were

loaded on shaved mice using harnesses as described by DenHollander and Allen (1985). The nymphs were allowed to fully engorge and detach. An eight day tick free period was followed by reharnessing and loading with six more nymphs which were also allowed to engorge completely. After a 21 day tick free period, mice were infested a third time with six nymphs for five days just prior to collecting spleens and performing the fusion to produce hybridomas.

Three mice were immunized in regimen III with Dermacentor andersoni SGA. Each mouse received five micrograms SGA by intravenous injection (IV) in the tail vein, on days 0, 14, 21, and 28. A final immunization containing five micrograms SGA was given IP three days prior to spleen collection and fusion.

#### Spleen Cell-Myeloma Cell Fusion

P3-NS1-Ag4-1 (abbreviated NS-1) mouse myeloma cells of BALB/c origin were obtained from the American Type Culture Collection (12301 Parklawn Drive, Rockville, MD). These cells were cultured in 25 cm<sup>2</sup> tissue culture flasks (Corning Glass Works, Corning, NY) in Roswell Park Memorial Institute medium (RPMI-1640) plus 15% fetal calf serum. Prior to fusion, myeloma cells were tested for sensitivity to the selective Hypoxanthine, Aminopterin, and Thymidine (HAT) medium. Eight flasks of NS-1 cells were pooled and washed twice in spleen collection medium (Appendix II) for each fusion.

Three mice were euthanized by cervical dislocation; their spleens were removed aseptically and placed in spleen collection medium on ice

for transport to the laboratory. Spleens were rinsed off with spleen collection medium, then either placed into a petri dish containing fresh spleen collection medium for manual cell extraction or a sterile plastic bag with five milliliters of medium for use in the Stomacher Lab Blender (Tekmar Company, Cincinnati, OH). Cell suspensions were filtered through several layers of sterile gauze into a sterile conical 15 ml centrifuge tube (Corning Glass Works, Corning, NY). Cells were pelleted by centrifugation at 150 x g for ten minutes, resuspended and washed three times in five milliliters of RPMI-1640 medium. Following the final wash, the spleen cells were resuspended in five milliliters of spleen cell collection medium and an aliquote was counted using Turk's solution (Appendix II) and a hemocytometer. Viability was assessed using Trypan blue (Appendix II).

The spleen cells were mixed with myeloma cells at an ideal ratio of 4:1 (spleen:myeloma) in a 50 ml conical centrifuge tube (Corning Glass Works, Corning, NY). Acceptable ratios for the cells can range from 1:1 to 10:1. The mixed cells were pelleted by centrifuging at 200 x g for five minutes. Supernatant was carefully aspirated from the pellet and the tube containing the pellet was placed into a 37 C waterbath, located in a laminar flow hood (Nuair, Inc., Plymouth, MN). Polyethylene glycol 1,300-1,600 (PEG - SigmaChemical Co., St. Louis, MO) was added at a ratio of one milliliter PEG to  $1.6 \times 10^8$  spleen cells over one minute. The cells were gently stirred for one more minute. Eight milliliters of growth medium (Appendix II) were added with slow swirling over the next four minutes. During this time a fusion factor was calculated by dividing the number of spleen cells by

$1.6 \times 10^8$ . Supernatant was decanted and cells were resuspended in a volume of growth medium equal to 22 ml x fusion factor. This suspension of cells was dispensed into 96 well microtiter plates (Corning Glass Works, Corning, NY) at approximately 100 microliters per well (two drops from a ten milliliter pipet). Only the center 60 wells were used per plate. Sterile water was dispensed into the outside rows of wells to maintain the humidity and decrease the chance of contamination. The plates were incubated at 37 C in 5% CO<sub>2</sub> overnight. The next day approximately 100 microliters of HAT medium (Appendix II) was added to each well.

#### Feeding and Visual Screening for Hybridoma Clones

Hybridoma feeding was accomplished by holding a sterile 18 gauge needle, hooked to an aspiration line, perpendicular to the microtiter plate and touching the point to the center of each well. This left approximately 100 to 150 microliters in each well. Fresh medium was added, two drops from a ten milliliter pipet per well.

On the second, third, fourth and seventh day after the fusion, HAT medium was added. On days 11, 14, 18, 22, and 26 post fusion HT medium (appendix) was added. Starting on day 28 growth medium was fed twice a week.

Every two to three days each well was observed, using an inverted microscope (Carl Zeiss Co., West Germany), for development of hybridoma clones. Clones were clusters of round cells which were larger and more refractile than other cells. Once cells from clones confluent

covered 50 -75% of the bottom of the microtiter well, medium from the well was screened for antibody to the tick immunogen by Dot-ELISA technique.

#### Dot-Enzyme Linked Immunosorbent Assay

This dot-enzyme linked immunosorbent assay (Dot-ELISA) technique was adapted from the method of Pappas et al. (1983). Disposable surgical gloves were worn during the preparation of the nitrocellulose disks to prevent protein contamination. A cardboard template and soft lead pencil were used to lightly draw circles on the nitrocellulose. Then one microliter of antigen, containing between 100 and 150 ng protein, was applied to the center of each circle. This resulted in a dot, approximately three millimeters (mm) in diameter, on the center of each disk. A light line was then drawn across each dotted circle with a number two lead pencil so that the top of the disk would be recognized throughout the procedure. Control disks were prepared the same way, except that one microliter of PBS was substituted for antigen in dotting. The nitrocellulose sheet was then dried at 56 C for ten minutes (Hawkes et al., 1982). The dotted circles were cut out using a 5 mm diameter paper punch and placed into wells of a 96 well microtiter plate using a spatula and a wooden applicator stick. These plates were ready for use but could be stored at 4 C for 90 days or frozen at -20 C over a 270 day period with no detectable loss in activity (Pappas et al., 1984). Antibody screening, of hybridoma clones or test animal sera, was performed by Dot-ELISA using immunogen dotted disks. All

unused protein binding sites on the disks were blocked by placing 75 microliters of 5% bovine serum albumin in 0.15 M phosphate buffered saline, pH 7.2, plus .05% tween 20 (BSA) in each microtiter well containing a dotted nitrocellulose disk. This plate was then incubated at room temperature for 20 minutes on a rotary mixer. Then all fluid was aspirated from each well using a Pasteur pipet connected to a suction line. Fifty microliters of 3% BSA was added to each disk followed by 100 microliters of medium (first antibody) from the corresponding well of the culture plate. The plate was again incubated at room temperature on the rotary shaker for 30 minutes. Following incubation all fluid was aspirated from the disks. Each well was washed three times with PBS - tween 20 using a Cornwall syringe (Becton Dickinson and Co., Oxnard, CA.) which dispensed approximately 300 microliters into each well. PBS - tween 20 was immediately aspirated in the first two washes, but was left on the disks for ten minutes for the last wash. All fluid was then aspirated and replaced with 50 microliters of second antibody, antisppecies IgG to the first antibody -- tagged with horseradish peroxidase (HRPO). A 30 minute incubation was followed by three washes, performed as before. A precipitable substrate, 4-Chloro-1-naphthal, was prepared fresh and 50 microliters was added to each disk. The disks were continuously observed during incubation with substrate and the reaction was stopped when the background color started to appear. This incubation never exceeded 30 minutes and was stopped by washing three times with PBS - tween 20 followed by aspirating all fluid and drying at 56 C for ten minutes with the lid off the plate.

Results were interpreted by first reading control disks. The PBS control disk had no visible dot while the positive control disk showed a distinct solid blue dot. All disks showing a solid blue dot were then called positive and subjectively graded on a scale of one to four plus relative to intensity of dots.

This technique was also used to titer the sera of animals that were immunized or infested. The only modifications involved performing doubling dilutions of the sera (first antibody) in a series of wells containing the dotted disks and 50 microliters of 1% BSA instead of 3% BSA. The titer was reported as the reciprocal of the dilution in the last well with a two plus reaction.

#### Cloning Hybridomas

As soon as positive wells are identified by antibody screening, the hybrid cells should be cloned to reduce the risk of overgrowth by cells not producing the desired antibody and to assure monoclonality (Goding, 1983; Campbell, 1984). Hybridoma cell lines should be cloned at least twice. The Poisson distribution is followed if small numbers of cells are grown. If 37% of the wells have no growth, it is a reasonable probability that those wells with growth are monoclonal (Goding, 1983). Limiting dilution is the method of cloning used by most people (Campbell, 1984), rather than cloning in soft agar. Limiting dilution cloning can be performed at various concentrations of hybridoma cells per well. Some authors refer to use of higher numbers of cells per well, such as five cells per well, as minicloning.

Thus the only difference between miniclone and formal clone is that in formal clone the goal is to obtain one hybridoma cell per three wells instead of five cells per well.

BALB/c mouse thymocytes were collected and used as feeder cells for both miniclones and formal clones. The thymus was aseptically removed from a three to four week old mouse and placed in five milliliters of spleen collection medium in a sterile petri plate held on ice. In a laminar flow hood connective tissue was removed and the spleen was transferred to five milliliters of fresh spleen collection medium in another sterile petri plate, where the thymocytes were teased out of the thymus. Thymocytes were pipetted through sterile gauze into a sterile 15 ml conical centrifuge tube. Filtered thymocytes were centrifuged at  $150 \times g$  for five minutes at  $4^\circ C$ , after which the supernatant was decanted. The thymocyte pellet was gently resuspended in five milliliters of growth medium and an aliquote counted. Hybridoma cells in the positive well were gently but thoroughly mixed with a pipet. A ten microliter aliquote was diluted 1:2 with trypan blue stain and viable cells were counted. For miniclones a sample volume calculated to contain 1000 cells, or in the case of a formal clone 65 cells, was added to ten milliliters of thymocyte suspension in growth medium containing at least  $10^6$  thymocytes per milliliter. This was well mixed and dispensed at 100 microliters per well into the center 60 wells of a microtiter plate. The plate was incubated at  $37^\circ C$  in 5%  $CO_2$  overnight. Then 100 microliters of growth medium was added to each well. Cells were refed with growth medium twice a week and observed

for clone development. Wells, in which cells confluenty covered 50 to 70% of the bottom, were screened by Dot-ELISA.

#### In Vitro Clone Expansion

At the same time wells were cloned; they were also expanded into the larger wells of a Falcon 24 well plate (Becton Dickinson and Co., Cxnard, CA). By pipetting 100 microliters of medium and hybridoma cells into a large well with 250 microliters of fresh growth medium expansion was possible without the need for thymocytes. If viable hybridoma cell numbers were low or more medium was added such that the cells were diluted more than 4:1 a feeder layer of thymocytes was added. Expansion into large wells produced an inoculum large enough to expand into 25 cm<sup>2</sup> flasks and also provided cells to be frozen, as described below. Cells were frozen for backup stock in case of either contamination or loss of required genetic material by the hybridoma cells to produce the desired specific antibody.

#### Freezing Hybridoma Cells

At each stage, and as early as possible, cells should be frozen. Cells collected from the original wells once the clones screened positive, and cells from expansion wells or flasks, were concentrated by centrifugation in 15 ml conical centrifuge tubes by centrifuging at 200 x g for 15 minutes at 4 C. The cells were resuspended in one

milli-liter of growth medium and 15% dimethyl sulfoxide or DMSO (Sigma Chemical Co., St. Louis, MO). The cell concentration was adjusted to  $4-6 \times 10^6$  cells / ml and one milliliter aliquotes were placed into sterile Nunc Cryotubes (Nunc Inter Med, Denmark). The cryotubes or freeze vials were placed into a styrofoam container at -70 C overnight and then transferred to a liquid nitrogen storage refrigerator (Union Carbide Corp., Danbury, CT) at -196 C. Spent medium from this process was used as first antibody in immunoblotting to characterize the antibody produced.

#### Reconstitution of Frozen Hybridoma Cells

The vial was removed from liquid nitrogen and immediately placed into a 37 C waterbath where it thawed within 60 seconds. Cells were diluted in ten milliliters of growth medium containing thymocyte feeder cells. This was dispensed at 200 microliters per well in a 96 well microtiter plate and incubated at 35 C in 5% CO<sub>2</sub>.

#### Ascites Production

Ascites production or in vivo expansion of clones was accomplished in syngeneic mice. The mice were injected with Pristane (Sigma Chemical Co., St. Louis, MO) 14 days prior to implanting the hybridoma clone. Hybridoma cells were harvested by centrifugation at 100 x g for 15 minutes in a conical centrifuge tube at 4 C. The medium was decanted and frozen at -20 C for future use in characterizing the antibody. The

cells were resuspended in a small volume of PBS and counted. The concentration was adjusted to  $1-2 \times 10^6$  cells / ml. One milliliter was injected IP into each mouse. Mice were observed daily for swelling of the abdomen. Four weeks after injection mice were tapped for the first of four times before they were euthanized. The abdomen of the mouse was disinfected with 70% ethanol. A sterile 18 guage needle was inserted no more than one centimeter (cm) into the abdomen while the mouse was on its back. Then the mouse was tilted so that the ascites dripped through the needle into a sterile 15 ml conical centrifuge tube. The ascites was rimmed with a wooden applicator stick and clarified by centrifuging at  $200 \times g$  for 15 minutes at 4 C to remove tumor cells and fibrin clots. Ascites was aliquoted and stored frozen at -20 C.

#### Purification of Monoclonal Antibody

Purification and concentration of ascites IgG was performed by the stepwise method of Bruck et al. (1986) using a DEAE Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA) column. Ascites was centrifuged at  $110,000 \times g$  for 30 minutes in a Beckman L8-70M Ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA) to remove cell debris and fibrin clots. The clarified ascites was dialyzed against column buffer (Appendix II) overnight at 4 C. Sixteen milliliters of DEAE Affi-Gel Blue which had been prewashed with acetate-NaCl-Isopropanol solution (Appendix II) was poured into a disposable 20 ml syringe barrel column. The column was then washed with ten bed volumes of prewash buffer

(Appendix II) and ten bed volumes of column buffer. The flow rate was adjusted to 30-40 ml / hour. Three milliliters of dialyzed ascites was loaded on the column. The column was eluted with three bed volumes of column buffer to remove transferrin. IgG was then eluted with three bed volumes of stepwise buffer, leaving albumin and proteases retained on the column.

The column was regenerated by washing with three bed volumes of 2 M guanidine hydrochloride (Appendix II) as recommended by Bio-Rad instead of 6 M guanidine hydrochloride used by Bruck et al. (1986). This was followed by ten bed volumes of prewash buffer and five bed volumes of column buffer.

The three bed volumes of stepwise buffer eluting the IgG was collected in two milliliter aliquotes which were screened for protein content by Beckman DB UV spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) at 280 nm. Seven, two milliliter, aliquotes were concentrated from 14 ml to approximately one milliliter by membrane ultrafiltration. One microliter of this was used as antigen on a nitrocellulose disk. A direct Dot-ELISA procedure utilizing goat-anti-mouse IgG (HRPO) diluted 1:100 as the antibody was performed to confirm the presence of mouse IgG.

#### Statistical Analysis

Multiple linear regression was performed by inputting data sets to the University of North Dakota mainframe computer using the STWMULT program. This program generated t values, that were the same as the

values which would be obtained using Dunnett's test (Williams, 1976), and levels of significance were reported. Dunnett's test compares the means for individual parameters of each treatment group to those of a control group. Statistical analysis was performed on data from all four immunization protocols and on the cell types microscopically identified ten micrometers from the attachment sites of ticks in immunization protocols I and II.

## RESULTS

### Antigens

All of the whole crude extracts used in this study had previously been shown to induce a degree of resistance to tick infestation. Ixodid tick antigens were prepared as described in the materials and methods section. Dermacentor andersoni ova extract prepared from the ova of three females yielded approximately three milliliters of extract with a protein content of 63 mg/ml. Salivary gland antigen (SGA) prepared from 12 adult, unfertilized, female D. andersoni, which had been allowed to engorge for four days, had a protein concentration of 1.5 mg/ml and a total volume of 0.5 ml. Forty unfed adult Amblyomma americanum were dissected and the guts collected to produce brush border fragments (BBF) with a protein content of 0.50 mg/ml and a total volume of 0.5 ml. The 27,000 x g supernatant, obtained as a byproduct of BBF preparation, had a protein concentration of 0.69 mg/ml and a total volume of one milliliter.

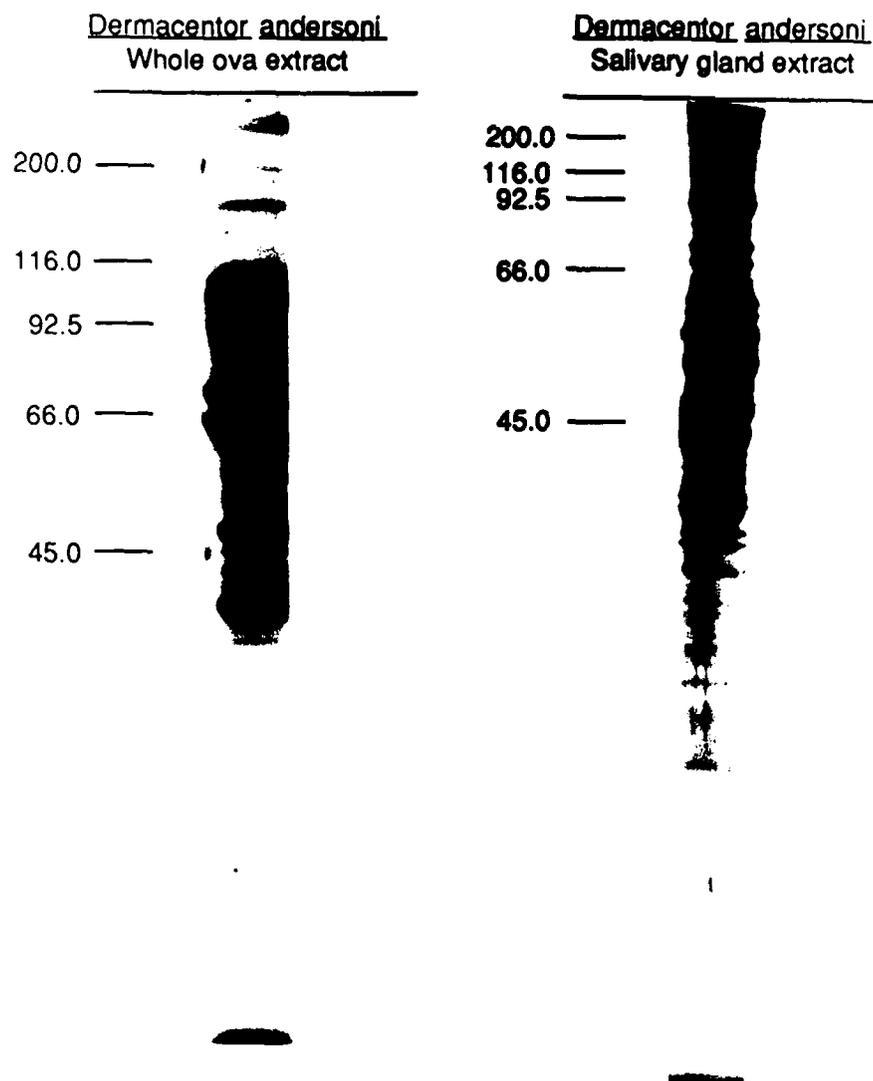
Following protein determination these antigens were then characterized by SDS-PAGE on 12% separating gels with samples run under reducing conditions at 30 ma until the running front reached 11.5 cm. Standard molecular weight markers were treated in the same manner and loaded on all gels at similar protein concentration as the samples being characterized. The molecular weight marker standards used were myosin (200 Kdal), beta-galactosidase (116 Kdal), phosphorylase B (92.5 Kdal), bovine serum albumin (66 Kdal), and ovalbumin (45 Kdal). Initial

gels were stained with buffalo black which has a sensitivity of 0.1 microgram protein while all other gels were stained with the more sensitive Morrissey silver stain procedure (Morrissey, 1981) capable of detecting one nanogram of protein.

Dermacentor andersoni whole ova extract and salivary gland antigen (SGA) lanes from SDS-PAGE gels, one buffalo black stained and one silver stained, are pictured in figure 1. The SGA pattern contains 31 distinct polypeptide bands, with molecular weights ranging from approximately 300 Kdal to less than ten Kdal; while 19 polypeptide bands are observed in the ova extract and have approximate molecular weights ranging from 292 Kdal to 36 Kdal. These numbers would be minimal numbers of polypeptide constituents, since any extract of a whole organ or tissue contains hundreds to thousands of different molecular constituents, many with similar molecular weights. Both D. andersoni whole ova extract and SGA were used in immunization regimens to develop hybridoma cells for producing monoclonal antibodies. Additionally these antigens were dotted to nitrocellulose disks used to screen hybridoma clones for antibody production. They were also electroeluted to nitrocellulose from SDS-PAGE gels for immunoblot characterization of antibodies from hybridoma clones and from immunized or infested animals.

Salivary glands are composed of many acini. These spherical structures are involved in production and secretion of many products as well as osmoregulation. Three distinct types, each composed of different cell and granule types have been characterized. Figure 2 is a transmission electron photomicrograph of a thin section of one type

Figure 1. SDS-PAGE gel lanes of Dermacentor andersoni antigens.

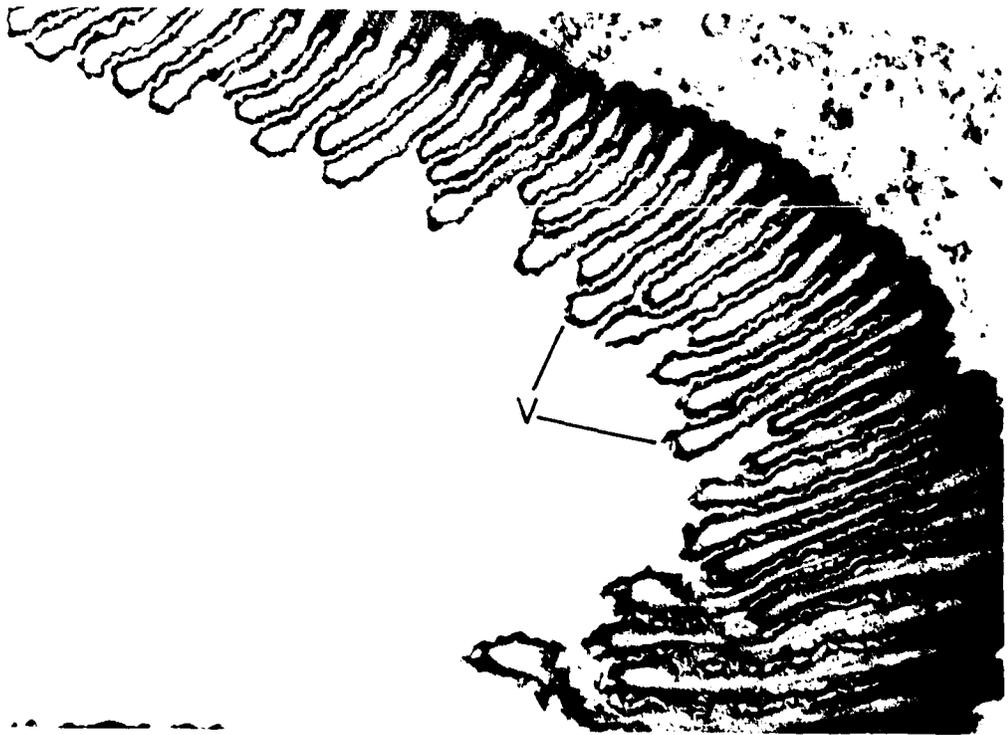
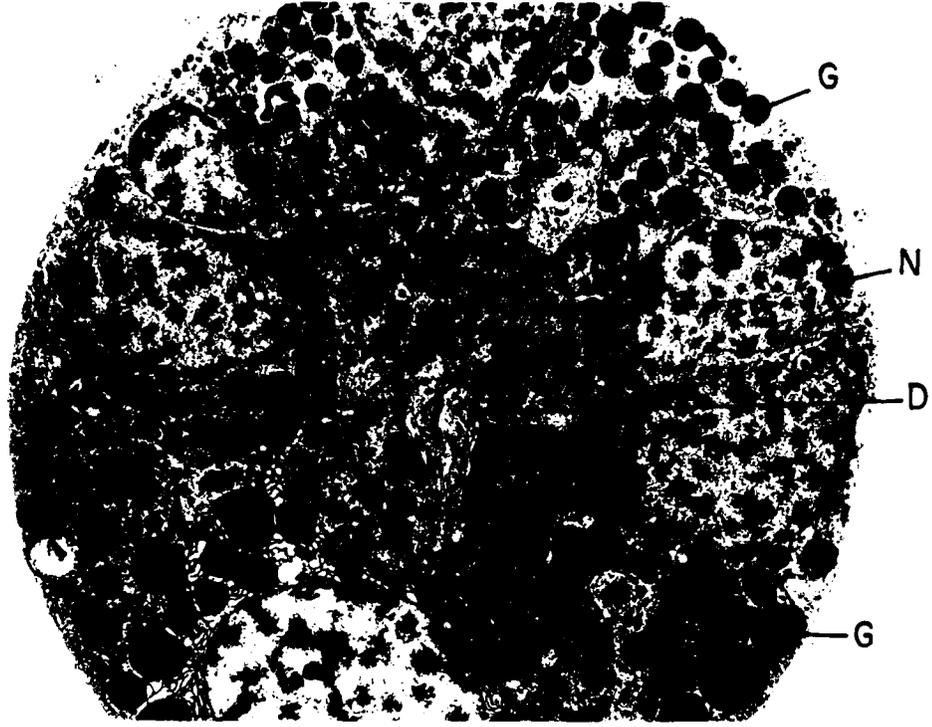


Note:

These lanes are from different SDS-PAGE gels but both were 12% separating gels run under reducing conditions at 30 milliamperes until the running front reached 11.5 cm. The ova extract lane, loaded with 45 micrograms protein, was stained with buffalo black and the SGA lane, loaded with ten micrograms protein, was silver stained. Position and weight in kilodaltons (Kdal) of standard molecular weight markers are marked on the left.

**Figure 2.** Transmission electron photomicrograph of a thin section of one type II acinus from the salivary gland of an unfed adult female Amblyomma americanum. Nuclei (N), granules (G), and ducts (D) are clearly visible in this section. In unfed adults, all six cell types found in type II acini contain granules (G). These cells contribute to the large array of polypeptides observed in SDS-PAGE gel patterns of salivary gland antigen (SGA). (original magnification: 2000 x)

**Figure 3.** Transmission electron photomicrograph of a section of Amblyomma americanum gut shows the villi (V) with surface microvilli, brush border. Villi are separated from the gut tissue in the preparation of the brush border fragment (BBF) antigen extract, used in immunization protocol IV. (original magnification: 15,000 x)



II acinus from the salivary gland of an unfed adult female Amblyomma americanum. Nuclei, granules, and ducts are clearly visible in this section. In unfed adults, all six cell types found in type II acini contain granules. These cells contribute to the large array of polypeptides observed in SDS-PAGE gel patterns of SGA.

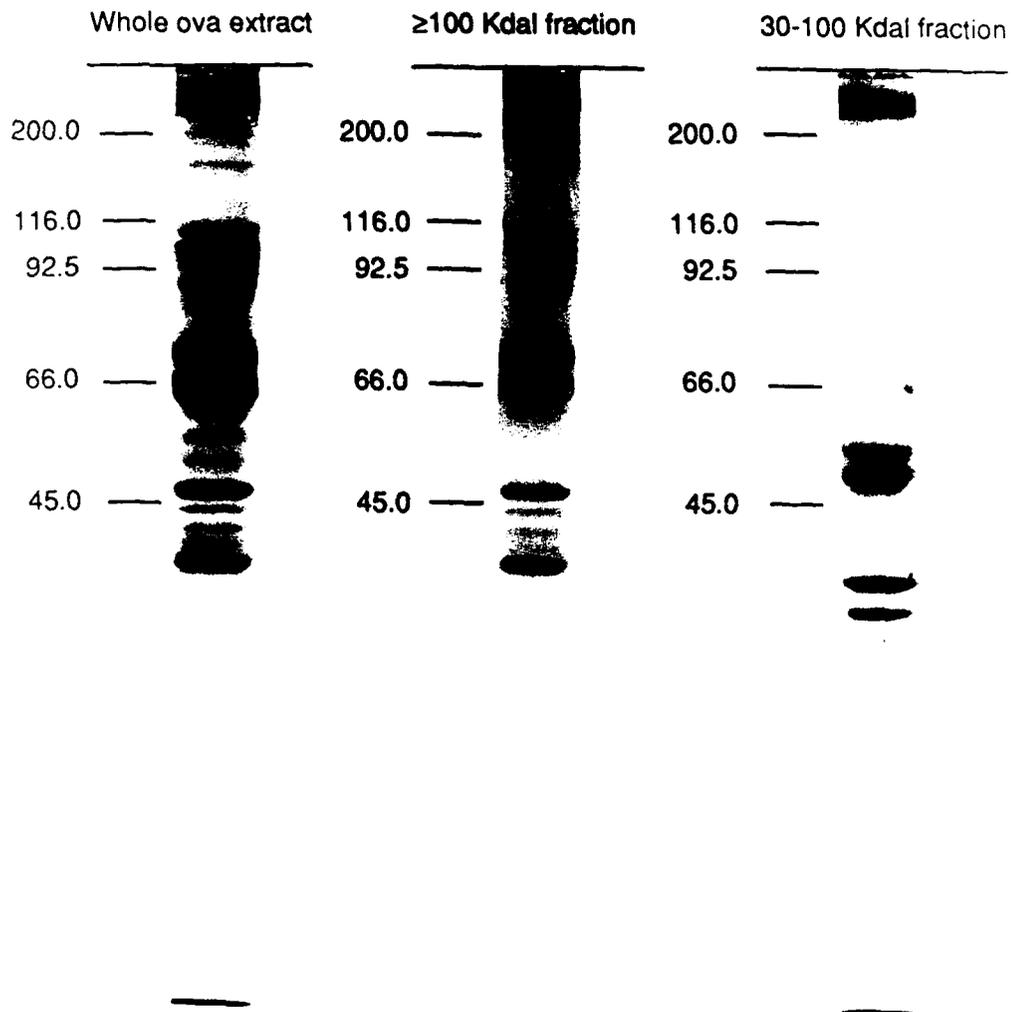
In the initial attempts to fractionate Dermacentor andersoni whole ova extract using Amicon ultrafiltration filters, described in detail in Materials and Methods, relatively large concentrations of protein, diluted in ten milliliters of PBS were filtered. In the first attempt, a sample containing 110 microliters of whole ova extract (4.73 mg protein) was loaded into the model 8010 chamber sequentially filtered through YM-100, XM-50, YM-30, and YM-10 filters at a nitrogen pressure of 25 psi. The retentate was concentrated to approximately one milliliter for each filter. The Bradford protein assay showed a total protein content of 3.03 mg was in the  $\geq 100$  Kdal fraction or YM-100 retentate. The protein contents of the other retenates were too low to determine using this assay. The SDS-PAGE analysis of D. andersoni whole ova extract and each of the retentates showed that the  $\geq 100$  Kdal fractions had the same pattern as the whole ova extract, while the 50-100 Kdal, 30-50 Kdal, and 10-30 Kdal fractions contained no detectable protein using buffalo black staining. Thus protein over loading and use of too great a nitrogen pressure led to protein trapping on the YM-100 filter.

Reducing the protein concentration to approximately 0.5 to 1.0 per cent and the nitrogen pressure to 10 psi for the YM-100 filter resolved some of the problems with protein trapping and separation of macro-

solutes. Some protein was still being lost, presumably to the filter, plastic ultrafiltration chamber, and pipets. Ultrafiltering 25 microliters of D. andersoni whole ova extract with a total protein content of 1.08 mg yielded 0.36 mg of  $\geq 100$  Kdal fraction, 0.12 mg of 30-100 Kdal fraction, and the 10-30 Kdal fraction was not detectable using the Bradford protein assay. The resulting  $\geq 100$  Kdal fraction is shown in lane two of figure 4. The 30-100 Kdal fraction appeared to be contaminated with higher molecular weight components. A second 30-100 Kdal fraction was ultrafiltered and then concentrated using a Centricon-30 micro concentrator for small volume concentration by Amicon. The SDS-PAGE gel pattern for this 30-100 Kdal product is shown in lane three of figure 4.

In figure 4, lane 1 contains D. andersoni whole ova extract that has polypeptide bands which range in molecular weight from 278 Kdal to 39.5 Kdal. Lane 2 contains the  $\geq 100$  Kdal ultrafiltration fraction of whole ova extract which has bands with the same molecular weight range as whole ova extract. Lane 3 contains the 30-100 Kdal ultrafiltration fraction of D. andersoni whole ova extract that has bands which range from 92.5 Kdal to 35.5 Kdal in molecular weight. Two, moderately dark adjacent bands, seen in figure 4, between the 66 Kdal and 45 Kdal markers of the whole ova extract lane, having approximate molecular weights of 58 Kdal and 53.5 Kdal, are totally missing in the  $\geq 100$  Kdal fraction lane. A relatively good fractionation was achieved with only a few very light bands observed above the 66 Kdal marker in the 30 to 100 Kdal fraction lanes. It is pertinent to point out that native proteins were subjected to ultra-filtration. These ultrafiltration

Figure 4. SDS-PAGE gel lanes of Dermacentor andersoni whole ova extract and ultrafiltration fractions.



Note:

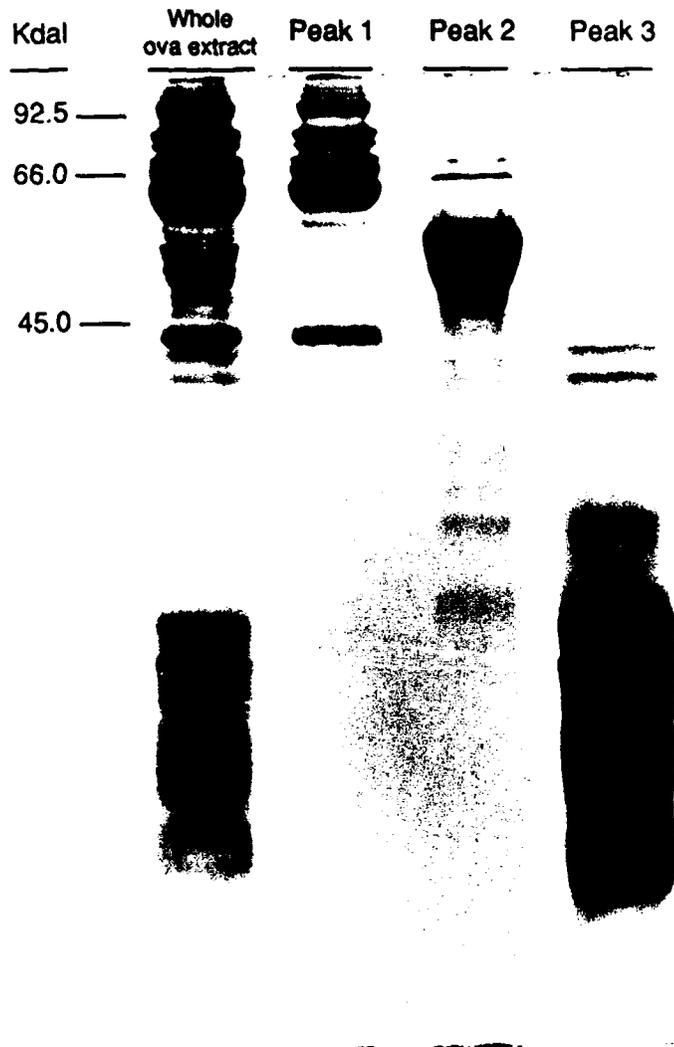
These lanes, stained with buffalo black, are from the same 12% separating gel run under reducing conditions at 30 miliamperes, until the running front reached 11.5 cm. Approximately 45 micrograms of protein were loaded per lane. Position and weight in kilodaltons (Kdal) of standard molecular weight markers are marked on the left.

fractions were then subjected to SDS-PAGE under reducing conditions. Thus bands with molecular weights below the ultrafiltration filter exclusion weight could represent subunits of native polypeptides. Ultrafiltration fractions, such as these, were used in skin testing of guinea pigs with different histories of tick exposure: guinea pig 2380, a tick naive control; guinea pig 2379, previously infested with D. andersoni nymphs; and guinea pig 2378, previously infested with D. andersoni larvae. (see Skin Testing, p. 100)

Amblyomma americanum whole ova extract is seen in lane 1 of the silver stained SDS-PAGE gel depicted in figure 5. Lanes 2, 3, and 4 contain peaks 1, 2, and 3 respectively from the Sephadex G-200 fractionation of A. americanum whole ova extract. Sephadex G-200 fractions (peaks 1, 2, and 3) of Amblyomma americanum whole ova extract used in this research were prepared by Valeria M. Howard, Ph.D. candidate. Sephadex G-200 peak 1 polypeptide bands, observed in a silver stained gel, had molecular weights ranging from 260 Kdal to 70 Kdal with a trailing band at 46 Kdal. Peak 2 polypeptide bands had molecular weights ranging from 67 Kdal to 53 Kdal. The molecular weights of polypeptide bands in peak 3 ranged from 45 Kdal down, with the heaviest concentration at 20 Kdal and below.

Amblyomma americanum whole ova extract was also fractionated by ultrafiltration and gave similar patterns to those seen with D. andersoni ova extract shown in figure 4. Fractions prepared by both methods were used to immunize guinea pigs which were then challenged with ticks to assess their ability to induce resistance to ixodid infestation. (see Guinea Pig Immunizations, p. 108)

**Figure 5. SDS-PAGE gel of *Amblyomma americanum* whole ova extract and fractions obtained by Sephadex G-200 gel filtration chromatography.**



**Note:**

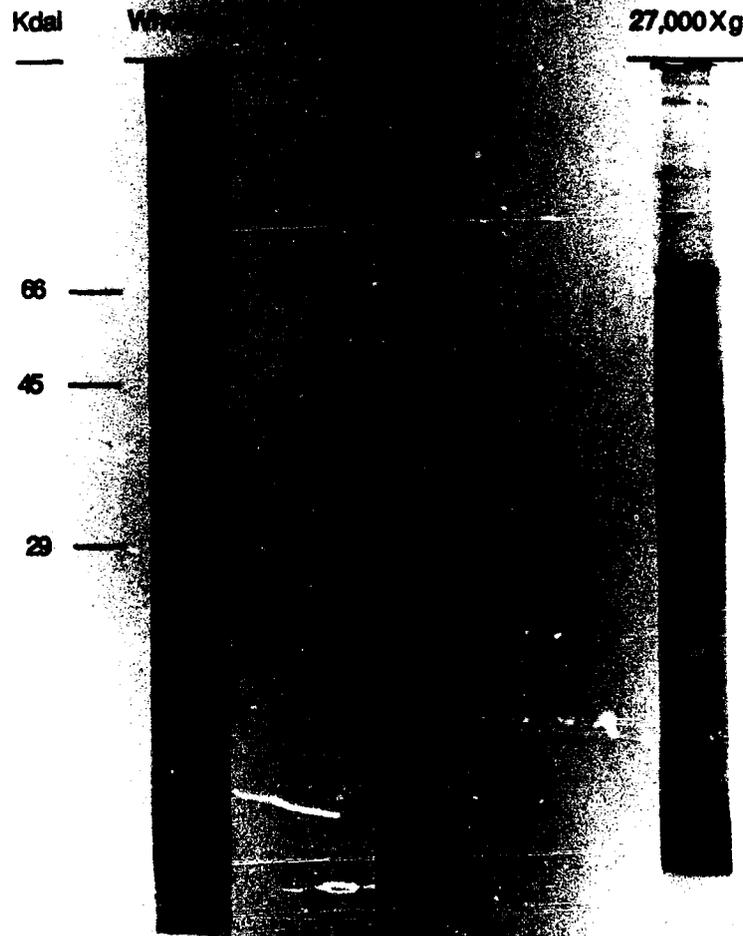
Silver stained, 12% separating gel, run under reducing conditions at 30 milliamperes until the running front reached 11.5 cm. Approximately ten micrograms of protein were loaded per lane. Position and weight in kilodaltons (Kdal) of standard molecular weight markers are marked on the left.

Amblyomma americanum whole gut was incorporated into immunizations with whole ova extract and Sephadex G-200 peaks 1, 2, and 3 of whole ova extract, in protocol III. Whole gut antigen fractionated by SDS-PAGE is seen in the first lane from the left in figure 6. Lanes 2 and 3 of figure 6 contain brush border fragments (BBF) and BBF preparation 27,000 x g supernatant, respectively. Note the strong similarity in these two gel patterns. Polypeptide bands in these patterns have molecular weights ranging from 132 Kdal to  $\leq$  10.4 Kdal. The BBF preparation appears to have more heavy polypeptide bands, above 66 Kdal, than the 27,000 x g supernatant which has more bands between 66 Kdal and 25 Kdal. All three of these preparations have a very intense band, of very light components, approaching the running front. Both BBF and 27,000 x g supernatant were utilized in immunization protocol IV. (See Guinea Pig Immunization, p. 108) A transmission electron photomicrograph of a section of Amblyomma americanum gut, figure 3, shows the villi with surface microvilli, brush border. Villi are separated from the remaining gut tissue in the preparation of the brush border fragment, BBF.

#### Skin Testing

Skin testing was performed to characterize the types of immune responses various ixodid ova extracts and ultrafiltration fractions of D. andersoni ova extract would elicit in previously exposed guinea pigs. Reactions that could occur following injection of an extract could be: immediate hypersensitivity, occurring within 30 minutes;

**Figure 6. Silver stained SDS-PAGE lanes of *Amblyomma americanum* whole body homogenates.**



**Note:**

These lanes are from the same 12% separating gel, run under reducing conditions at 50 mA for 1.5 h until the running front reached 11.5 cm. Approximately ten micrograms of protein were loaded per lane. Position and weight in kilodaltons (Kdal) of standard molecular weight markers are marked on the left.

arthus reaction, peaking at six hours; and/or delayed type hypersensitivity which peaks at 24 hours. The type of reaction gives clues to host mechanisms involved, and it relates to the type of damage that may occur at tick feeding sites. Antigens used for immunization must be immunogenic, but extensive skin or tissue damage is undesirable. The skin testing was performed on three guinea pigs, each with a different history of tick exposure: guinea pig 2380, a tick naive control; guinea pig 2379, previously infested with D. andersoni nymphs; and guinea pig 2378, previously infested with D. andersoni larvae.

The area of the reaction to each antigen preparation and an indication of erythema and/or induration are listed in table 3. The 30 minute reactions, looking for immediate hypersensitivity, were not observed on the control guinea pig, 2380, as the other animals were still being injected. For this reason, and because the animals were not syngeneic, the 30 minute reactions will be compared only to the reaction to PBS, base line reaction, in the same animal. Guinea pig 2379 had an immediate hypersensitivity reaction to only one antigen, D. andersoni 30-100 Kdal fraction which had a reaction area of 0.90 cm<sup>2</sup>. All other reactions were smaller than or the same size as the PBS reaction of 0.58 cm<sup>2</sup>. Guinea pig 2378 had an immediate hypersensitivity reaction to every antigen except two, D. andersoni whole ova extract and D. andersoni 50-100 Kdal fraction. Dermacentor andersoni ≥ 100 Kdal fraction (antigen 4 in table 3), at 1.88 cm<sup>2</sup>, and D. andersoni 30-100 Kdal fraction, at 1.70 cm<sup>2</sup>, were the antigens eliciting the strongest immediate hypersensitivity reactions in this animal, previously infested by D. andersoni larvae. It is interesting that the

**Table 3. The area of skin test reactions to ixodid ova extracts.**

Ova extract	area in cm <sup>2</sup> at hours post injection					
	Guinea pig #	0.5	1	6	24	48
1. <u>Dermacentor andersoni</u> whole ova extract	2380	nd	0.60	0.54	1.07‡	0.56
	2379	0	0.67	1.29	2.27‡	0.75‡
	2378	0.77	0	0.97	1.57‡	1.14‡
2. <u>Dermacentor andersoni</u> ≥ 100 Kdal fraction	2380	nd	0.82	0.48	0.76	0.50
	2379	0.38	0.40	0.59	0.42	0.27
	2378	1.88	0.87	1.33	1.27*	0.60
3. <u>Dermacentor andersoni</u> 30-100 Kdal fraction	2380	nd	0.45	0.21	0.46	0.24
	2379	0.90	0	0.59	0.80	0.58
	2378	1.70	0.95	0.44	0.83*	0.86
4. <u>Dermacentor andersoni</u> ≥ 100 Kdal fraction	2380	nd	0.44	0.37	2.20	1.65
	2379	0.37	0.47	1.38	3.20††*	2.94††*
	2378	1.30	0.91	2.64	4.48††*	4.77††*
5. <u>Dermacentor andersoni</u> 50-100 Kdal fraction	2380	nd	0.49	1.06	1.64	0.58
	2379	0.44	0.27	0.28	1.29	0.71*
	2378	0.86	0.33	0.55	1.37	0.60‡
6. <u>Dermacentor albipictus</u> whole ova extract	2380	nd	0.50	0.19	1.86	0.20
	2379	0.45	0.69	0.61	1.18*	0.78*
	2378	1.46	1.20	1.46	2.31	1.40‡
7. <u>Amblyomma americanum</u> whole ova extract	2380	nd	0.67	0.68	0.91	0.44
	2379	0.59	0.66	0.79	0.99*	0.66*
	2378	1.38	1.12	0.37	1.38	1.42‡
8. PBS control	2380	nd	0.55	0.21	0	0
	2379	0.58	0.49	0.18	0	0
	2378	0.95	0.90	0.29	0	0

**Note :**

Skin test consisted of intradermal injection of 20 micrograms of either whole or ultrafiltration fractions of ixodid ova extracts in a volume of 100 microliters of 0.15 M phosphate buffered saline, pH 7.4.

Testing was on male Hartley guinea pigs with different tick exposure histories: guinea pig 2380, a tick naive control; guinea pig 2379, previously infested with Dermacentor andersoni nymphs; and guinea pig 2378 previously infested with Dermacentor andersoni larvae.

nd = Not determined; Erythema = ‡; Induration = \*; and White ulcerated center = †.

whole ova extract from the homologous tick species previously infesting this guinea pig did not elicit an immediate reaction but whole ova from two other species, D. albipictus and A. americanum, did cause immediate reactions. One possible explanation is that circulating antibody to epitopes unique to D. andersoni whole ova extract complexed with the antigen and prevented IgE-antigen interaction.

By six hours the PBS reaction sites had decreased in size, indicating the buffer vehicle caused no reaction, but were too small for comparison with the reactions of the antigens. Therefore the six hour and 24 hour reactions were compared to those found in the tick naive control, guinea pig 2380. Some nonspecific or cross reactive response must have been stimulated, because the tick naive control reactions, at a minimum, are all nearly twice the size of the PBS reaction at six hours except for two antigens, D. andersoni 30-100 kdal fraction and D. albipictus whole ova extract. Both previously exposed guinea pigs reacted to the antigens with either an Arthus or early delayed response except for the 50-100 Kdal fraction and guinea pig 2378 did not react to A. americanum whole ova extract at six hours.

At 24 hours post injection, the reactions all intensified and most had peaked with the exception of the second antigen in table 3, D. andersoni  $\geq 100$  Kdal fraction, which peaked at six hours. Peak reactions at 24 hours are characteristic of delayed type hypersensitivity reactions. When compared to the naive control reactions, delayed type hypersensitivity responses appear to have occurred in guinea pig 2378 to all antigens except D. andersoni  $\geq 100$  Kdal, antigen 2, and D. andersoni 50-100 Kdal fraction. Guinea pig 2379 evaluated by this

criteria would have a delayed hypersensitivity reaction only to D. andersoni whole ova extract, 30-100 Kdal fraction, and  $\geq$  100 Kdal fraction (antigen 4 in table 3). The presence of induration at 24 hours and comparison of the reactions with the same antigen test sites at earlier readings would contradict this interpretation and add all of the antigens used to this list except for D. andersoni  $\geq$  100 Kdal fraction (antigen 2 in table 3). Antigen 4 in table 3, D. andersoni  $\geq$  100 Kdal fraction, stimulated the strongest response, in the previously exposed animals, having the reactions with the largest area, intense erythema, induration, and white ulcerated centers. These reactions contrasted with the reactions to antigen 2 in table 3, another batch of D. andersoni  $\geq$  100 Kdal fraction, showed significant differences in the degrees of reaction stimulated.

Biopsies were taken from each skin test site on all test animals after the last reading at 48 hours post antigen administration. The tissue was placed in Karnovsky's fixative, processed, sectioned, and stained with Wolbach's geimsa modification. Infiltrating hematogenous cells were identified and counted in three oil immersion fields located at 45 degrees to the dermal-epidermal junction. This process was repeated at each of five distances from the dermal-epidermal junction: at the junction, 10, 20, 30, and 40 micrometers from that point. Where possible, the cells in three oil immersion fields were counted. Table 4 contains a composite of all the cells identified in each section, which is a mean plus or minus the standard deviation calculated for 12 to 15 oil immersion fields. The dominant infiltrating cell type in almost every case was the eosinophil.

Table 4. The cellular infiltrate at skin test sites.

Ova extract	Mean $\pm$ SD of cell types counted in each section.					
	Guinea pig #	Eosinophils	Neutrophils	Lymphoid cells	Basophils	Mast cells
1. <u>Dermacentor andersoni</u> whole ova extract	2380	0.5 $\pm$ 0.7	0.4 $\pm$ 0.7	0	0.1 $\pm$ 0.4	0.1 $\pm$ 0.3
	2379	3.1 $\pm$ 1.7	0.2 $\pm$ 0.6	0.8 $\pm$ 1.5	0	0.1 $\pm$ 0.5
	2378	4.8 $\pm$ 8.5	0.3 $\pm$ 0.6	0	0.1 $\pm$ 0.3	0
2. <u>Dermacentor andersoni</u> $\geq$ 100 Kdal fraction	2380	3.0 $\pm$ 3.1	0.1 $\pm$ 0.3	0.1 $\pm$ 0.3	0.1 $\pm$ 0.3	0.1 $\pm$ 0.3
	2379	0.5 $\pm$ 0.9	0	0.4 $\pm$ 0.6	0	0
	2378	2.1 $\pm$ 1.9	0	11.3 $\pm$ 7.0	0.1 $\pm$ 0.4	0.2 $\pm$ 0.4
3. <u>Dermacentor andersoni</u> 30-100 Kdal fraction	2380	0	0.1 $\pm$ 0.3	0	0	0.1 $\pm$ 0.3
	2379	15.5 $\pm$ 8.0	0	0.1 $\pm$ 0.3	0	0.1 $\pm$ 0.3
	2378	0.8 $\pm$ 0.3	0	7.9 $\pm$ 5.7	0	0.2 $\pm$ 0.4
4. <u>Dermacentor andersoni</u> $\geq$ 100 Kdal fraction	2380	2.3 $\pm$ 3.6	0.1 $\pm$ 0.3	0	0.1 $\pm$ 0.3	0.3 $\pm$ 0.8
	2379	47.0 $\pm$ 11.3	0	0.1 $\pm$ 0.3	0	0.2 $\pm$ 0.4
	2378	4.2 $\pm$ 8.7	0.1 $\pm$ 0.3	3.5 $\pm$ 2.7	0	0.1 $\pm$ 0.3
5. <u>Dermacentor andersoni</u> 50-100 Kdal fraction	2380	nd	nd	nd	nd	nd
	2379	28.1 $\pm$ 16.1	0	3.3 $\pm$ 2.0	0	0.3 $\pm$ 0.6
	2378	13.9 $\pm$ 7.6	0	1.2 $\pm$ 1.2	0	0
6. <u>Dermacentor albipictus</u> whole ova extract	2380	9.2 $\pm$ 2.6	0.2 $\pm$ 0.4	0.1 $\pm$ 0.3	0.0 0.7	0.3 $\pm$ 0.5
	2379	12.0 $\pm$ 5.0	0	1.7 $\pm$ 2.5	0	0
	2378	1.0 $\pm$ 0.7	0	5.7 $\pm$ 9.1	0	0
7. <u>Amblyomma americanum</u> whole ova extract	2380	35.1 $\pm$ 32.0	0.1 $\pm$ 0.5	0.3 $\pm$ 0.8	0.1 $\pm$ 0.3	0.1 $\pm$ 0.3
	2379	9.2 $\pm$ 7.0	0	0.1 $\pm$ 0.3	0.1 $\pm$ 0.3	0.2 $\pm$ 0.4
	2378	3.1 $\pm$ 3.4	0.1 $\pm$ 0.3	1.0 $\pm$ 0.8	0	0.2 $\pm$ 0.4
8. PBS control	2380	nd	nd	nd	nd	nd
	2379	0.2 $\pm$ 0.6	0.8 $\pm$ 0.3	0.4 $\pm$ 0.9	0	0
	2378	0.1 $\pm$ 0.3	0.1 $\pm$ 0.3	0.3 $\pm$ 0.8	0	0

## Note :

Seven micrometer thick paraffin sections of biopsies taken from skin test sites were stained with Wolbach's geimsa. Cells were counted in one, two, or three oil immersion fields at five distances from the epidermis: at the epidermis, 10, 20, 30, and 40 micrometers. Twelve to fifteen oil immersion fields were counted for each value listed.

Although the dominant cell type observed in nearly every section was the eosinophil and eosinophils and lymphoid cells (lymphocytes and/or plasmacytes) were the only cells observed in significant numbers, some patterns can be detected. Guinea pig 2380, tick naive control, had only slight cellular infiltrate which was dominated by eosinophils. This guinea pig had fewer eosinophils in every section except for the one from the Amblyomma americanum whole ova extract test site which had more eosinophils, a mean of 35.1, when compared to the guinea pig previously infested with nymphs. This test site also had a wide standard deviation. Guinea pig 2379, previously infested with nymphs, reacted to most antigens with the largest infiltration of eosinophils observed in the three animals. Guinea pig 2378, previously infested with larvae, generally had fewer eosinophils with their numbers being very similar to those found in the naive control sections. Guinea pig 2378 was the only animal that had significant numbers of lymphoid cells in sections. At antigen test sites for D. andersoni  $\geq$  100 Kdal fraction, antigen 2, D. andersoni 30-100 Kdal fraction, and D. albipictus whole ova extract the reaction contained more lymphoid cells than eosinophils; while at D. andersoni  $\geq$  100 Kdal fraction, antigen 4, lymphoid cells and eosinophil numbers were nearly the same.

Guinea Pig Immunizations

## Evaluation of Induced Resistance

In immunization protocol I, pairs of guinea pigs were immunized with either ten micrograms of A. americanum whole ova extract per 250 g body weight, 7.5 micrograms of  $\geq 100$  Kdal ultrafiltration fraction per 250 g body weight, or five micrograms of 30-100 Kdal ultrafiltration fraction of A. americanum whole ova extract per 250 g body weight to induce resistance to tick infestation. Seven days after the final immunization the animals were challenged with 100 A. americanum larvae for five days. Table 5 illustrates the effect of the immunization on the number of larvae which engorged; the average weight of larva after a five day infestation; and the per cent of larvae which were viable or able to molt to the nymph stage.

For immunization protocol I, statistical evaluation of engorgement revealed that those guinea pigs immunized with whole ova extract (0.05 level) and 30-100 Kdal fraction (0.05 level), as well as the two exposure control (0.01 level) had significantly fewer ticks engorge than the one exposure controls. There was no statistical difference at the 0.05 level between the number of ticks engorging on guinea pigs immunized with  $\geq 100$  Kdal fraction and one exposure controls. Although some resistance was induced in animals immunized with whole ova extract and 30-100 Kdal fractions it was much less than acquired by two exposure controls, as both treatment groups had significantly more ticks engorge (0.01 level) than the two exposure controls. Mean

**Table 5. Effect of immunizing pairs of guinea pigs, with ultrafiltration fractions of Amblyomma americanum ova, on infesting Amblyomma americanum larvae engorgement and viability (protocol I).**

Immunization or treatment	% Engorged ( mean $\pm$ SD )			Larval weight ( mean $\pm$ SD ) $\times 10^{-4}$ g	% Viable ( mean $\pm$ SD )
	Full	Partial	Unengorged		
Whole ova extract	80.6 $\pm$ 0.8	4.5 $\pm$ 2.4	14.8 $\pm$ 3.3	7.08 $\pm$ 0.27	21.95 $\pm$ 0.77
$\geq$ 100 Kdal fraction	89.1 $\pm$ 3.3	1.4 $\pm$ 2.0	9.5 $\pm$ 5.3	7.52 $\pm$ 0.76	26.72 $\pm$ 15.58
30-100 Kdal fraction	81.5 $\pm$ 6.6	4.5 $\pm$ 3.9	14.0 $\pm$ 2.7	6.82 $\pm$ 0.41	15.69 $\pm$ 11.94
Two exposure control	13.9 $\pm$ 4.2	11.9 $\pm$ 7.1	74.1 $\pm$ 18.4	2.26 $\pm$ 0.27	8.83 $\pm$ 6.68
One exposure control	100.0 $\pm$ 0	0	0	10.40 $\pm$ 0.69	55.60 $\pm$ 0.34

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Note:

Immunization dosage rate per 250 grams body weight was: ten micrograms for the whole ova extract, 7.5 micrograms for the  $\geq$ 100 kilodalton fraction, and five micrograms for the 30-100 Kdal fraction. Challenge infestation was with 100 Amblyomma americanum larvae.

engorgement weights for ticks from the three immunization treatment groups were statistically less (0.05 level) than those from the one exposure controls and greater (0.01 level) than those from the two exposure controls. Only the 30-100 Kdal fraction immunized guinea pigs (0.05 level) and the two exposure controls (0.01 level) had significantly fewer ticks molt (viable) than the one exposure controls. This indicates that resistance was acquired by the two exposure controls and a lower level of resistance was induced in the guinea pigs immunized with 30-100 Kdal fraction of A. americanum whole ova extract.

Immunization protocol II involved immunizing pairs of guinea pigs with Sephadex G-200 peaks of A. americanum whole ova extracts, whole ova extract, or phosphate buffered saline (PBS). The data obtained from immunization protocol II can be seen in Table 6. Immunization protocol II had little or no effect on the amount of blood engorged by A. americanum larvae. There were no statistically significant differences between the mean larval weights of the different treatment groups and the one exposure controls. The two exposure controls had significantly fewer fully engorged larvae (0.01 level) than any other pair of guinea pigs. Compared to the one exposure controls, the PBS controls (0.05 level) and peak 3 immunized guinea pigs (0.05 level) had significantly more fully engorged larvae (0.05 level) than the other treatment groups and controls. The number of fully engorged ticks from guinea pigs immunized with peak 1 and peak 2 were not significantly different from those obtained from the one exposure controls. The two exposure animals had significantly more partially engorged larvae when compared to the one exposure controls (0.01 level).

**Table 6. Effect of immunizing pairs of guinea pigs, with Sephadex G-200 peaks of Amblyomma americanum ova, on infesting Amblyomma americanum larvae engorgement and viability (protocol II).**

Immunization or treatment	% Engorged ( mean $\pm$ SD )			Larval weight ( mean $\pm$ SD ) $\times 10^{-4}$ g	% Viable ( mean $\pm$ SD )
	Full	Partial	Unengorged		
Whole ova extract	68.1 $\pm$ 6.1	14.4 $\pm$ 0.8	5.5 $\pm$ 2.6	7.49 $\pm$ 0	41.80 $\pm$ 2.55
Peak 1	61.4 $\pm$ 1.1	21.1 $\pm$ 0.4	2.5 $\pm$ 1.8	7.96 $\pm$ 0.03	56.57 $\pm$ 4.12
Peak 2	67.7 $\pm$ 10.6	5.3 $\pm$ 1.2	5.9 $\pm$ 1.2	7.49 $\pm$ 0.53	22.63 $\pm$ 19.02
Peak 3	82.7 $\pm$ 3.0	3.7 $\pm$ 0.8	3.2 $\pm$ 0.1	8.04 $\pm$ 0.22	50.24 $\pm$ 6.49
PBS control	78.4 $\pm$ 3.0	5.1 $\pm$ 1.4	2.6 $\pm$ 0.7	7.13 $\pm$ 0.43	47.19 $\pm$ 4.69
Two exposure control	27.6 $\pm$ 3.0	25.9 $\pm$ 8.1	10.8 $\pm$ 6.3	4.99 $\pm$ 0.83	27.64 $\pm$ 3.02
One exposure control	59.1 $\pm$ 0.7	12.9 $\pm$ 3.4	5.5 $\pm$ 3.0	7.72 $\pm$ 0.81	48.64 $\pm$ 7.60

Note:

Immunization dosage rate was 50 micrograms per 400 grams body weight.  
Challenge infestation was with 100 Amblyomma americanum larvae.

Resistance was acquired by the two exposure controls which had significantly fewer larvae molt to nymphs (0.05 level) than one exposure controls. Apparently resistance was induced in animals immunized with Sephadex G-200 peak 2 of A. americanum whole ova extract, which had significantly fewer larvae molt to nymphs (0.01 level) than one exposure controls. It may be important to note the variation in percent of viable ticks in the peak 2 immunized pair which ranged from 10.2 to 36 as compared to two exposure controls which had a range of 25.5 to 29.8 and the one exposure controls which had a range of 43.3 to 54.0.

Immunization protocol III data is depicted in tables 7 and 8. For this protocol, pairs of guinea pigs were immunized with 20 micrograms of the appropriate Sephadex G-200 peaks of A. americanum whole ova extract plus 20 micrograms whole gut antigen per 250 g body weight and challenged with four pair of A. americanum adults. There were no significant differences detected: in number of days engorging, with means ranging from 18.75 to 24.87 days; percent of ticks that engorged, with means ranging from 62.5 to 100 percent; engorged tick weight, with means ranging from 0.08 to 0.36 grams; egg mass weight, with means ranging from 0.04 to 0.17 grams; nor percent hatch, with means ranging from 30 to 93.5 percent.

Immunization protocol IV included immunizing pairs of guinea pigs with either one microgram of brush border fragments (BBF) per 250 g body weight or ten micrograms of 27,000 x g supernatant per 250 g body weight. Only one control animal was included and it was immunized with PBS. The animals were challenged with four pairs of A. americanum

**Table 7. The impact of immunization protocol III on tick engorgement and survival.**

Immunization or treatment	number of days engorging (mean $\pm$ SD)	percent engorging (mean $\pm$ SD)	engorged tick weight (mean $\pm$ SD) in grams	percent nonviable (mean $\pm$ SD)
Whole ova extract plus gut	19.75 $\pm$ 7.22	100.0 $\pm$ 0.0	0.10 $\pm$ 0.05	12.5 $\pm$ 18.0
Peak 1 plus gut	20.66 $\pm$ 5.89	75.0 $\pm$ 35.0	0.08 $\pm$ 0.09	0
Peak 2 plus gut	19.16 $\pm$ 4.28	75.0 $\pm$ 0.0	0.17 $\pm$ 0.16	0
Peak 3 plus gut	20.57 $\pm$ 6.14	100.0 $\pm$ 0.0	0.12 $\pm$ 0.09	37.5 $\pm$ 18.0
Gut only	20.75 $\pm$ 6.40	62.5 $\pm$ 18.0	0.36 $\pm$ 0.30	12.5 $\pm$ 18.0
Two exposure control	18.75 $\pm$ 5.57	100.0 $\pm$ 0.0	0.16 $\pm$ 0.13	0
One exposure control	24.87 $\pm$ 5.49	87.5 $\pm$ 18.0	0.10 $\pm$ 0.12	12.5 $\pm$ 18.0

**Note:**

Pairs of guinea pigs were immunized with 20 micrograms of antigen; whole ova, peak 1, peak 2, or peak 3, plus 20 micrograms of Amblyomma americanum gut antigen per 250 g body weight. Gut only controls received only 20 micrograms of gut antigen per 250 g body weight. The challenge infestation was two pair of adult Amblyomma americanum per ear per animal.

**Table 8. The impact of immunization protocol III on tick engorgement and reproduction.**

Immunization or treatment	number of days engorging (mean $\pm$ SD)	engorged tick weight (mean $\pm$ SD) in grams	egg mass weight (mean $\pm$ SD) in grams	estimated ave. % hatch (mean $\pm$ SD)
Whole ova extract plus gut	19.75 $\pm$ 7.22	0.10 $\pm$ 0.05	0.04 $\pm$ 0.03	50.0 $\pm$ 70.0
Peak 1 plus gut	20.66 $\pm$ 5.89	0.08 $\pm$ 0.09	0.06 $\pm$ 0.05	40.5 $\pm$ 56.0
Peak 2 plus gut	19.16 $\pm$ 6.14	0.17 $\pm$ 0.16	0.11 $\pm$ 0.06	75.0 $\pm$ 21.0
Peak 3 plus gut	20.57 $\pm$ 4.28	0.12 $\pm$ 0.09	0.06 $\pm$ 0.08	30.0 $\pm$ 42.0
Gut only	20.75 $\pm$ 6.40	0.36 $\pm$ 0.30	0.17 $\pm$ 0.14	70.0 $\pm$ 0.0
Two exposure † control	18.75 $\pm$ 5.57	0.16 $\pm$ 0.13	0.07 $\pm$ 0.08	93.5 $\pm$ 5.0
One exposure control	24.87 $\pm$ 5.49	0.10 $\pm$ 0.12	0.12 ††	50.0

**Note:**

Pairs of guinea pigs were immunized with 20 micrograms of antigen; whole ova, peak 1, peak 2, or peak 3, plus 20 micrograms of Amblyomma americanum gut antigen per 250 g body weight. Gut only controls received only 20 micrograms of gut antigen per 250 g body weight.

The challenge infestation was two pair of adult Amblyomma americanum per ear per animal.

Only partially or fully engorged viable female ticks were weighed and averaged.

Egg mass weight was the total weight of all eggs laid divided by the number of ticks that laid eggs.

†. Due to the long engorgement period, the two exposure guinea pigs were only infested once.

††. Only one tick became fully engorged and laid eggs from this pair of guinea pigs.

adults. The data for immunization protocol IV is shown in table 9. Comparing only the tick data from BBF immunized animals to those from the PBS control guinea pig revealed no statistically significant differences between these groups. Comparing only the tick data from the 27,000 x g supernatant immunized guinea pigs to those from the PBS immunized control showed significant differences in tick weight (0.05 level) and egg mass weight (0.01 level). Immunizing with one microgram of BBF per 250 g body weight statistically gave no significant difference in induced tick resistance when compared to the PBS immunized control. The wide variation observed in data from this pair of guinea pigs may mask potentially significant differences in tick weights and egg mass weights. Immunizing with ten micrograms of 27,000 x g supernatant per 250 g body weight induced resistance which was statistically significant in tick engorged weight (0.05 level) and egg mass weight (0.01 level). Additionally 50% of the female ticks that engorged on the guinea pigs immunized with 27,000 x g supernatant died, while none of the ticks engorging on the PBS control or the BBF immunized guinea pigs died.

#### Histology

The histology of tick attachment sites was studied for immunization protocols I and II where the challenge infestations were 100 A. americanum larvae per animal. One punch biopsy, including attached tick, was taken from each animal. The biopsy was immediately placed into cold Karnovsky's fixative. The tissue was processed, sectioned

**Table 9. Effect of immunizing pairs of guinea pigs with Amblyomma americanum brush border fragments (BBF) or 27,000 x g supernatant on adult female Amblyomma americanum engorgement and reproduction (protocol IV).**

Guinea pig number	Immunization	Ave. # days engorging	Ave. tick weight (in grams)	Ave. egg mass weight (in grams)	% Hatch
2547	BBF	14.75 ± 3.09	0.07 ± 0.03	0.02 ± 0.02 (2)	65 ± 35
2458	BBF	13.50 ± 0.58	0.34 ± 0.24	0.16 ± 0.12 (4)	44 ± 39
2459	27,000 x g	14.25 ± 0.50	0.11 ± 0.11	0.08 (1)	99
2460	27,000 x g	16.25 ± 4.35	0.10 ± 0.13	0.05 ± 0.07 (2)	30 ± 28
2462	PBS	16.50 ± 2.38	0.24 ± 0.06	0.09 ± 0.03 (4)	38 ± 25

**Note:**

All female ticks were weighed.

Average egg mass weight equals total egg mass divided by number of ticks laying eggs, given in parenthesis.

\* Three of the four ticks died; one fully engorged died two weeks after detaching.

† One tick died attached to the host.

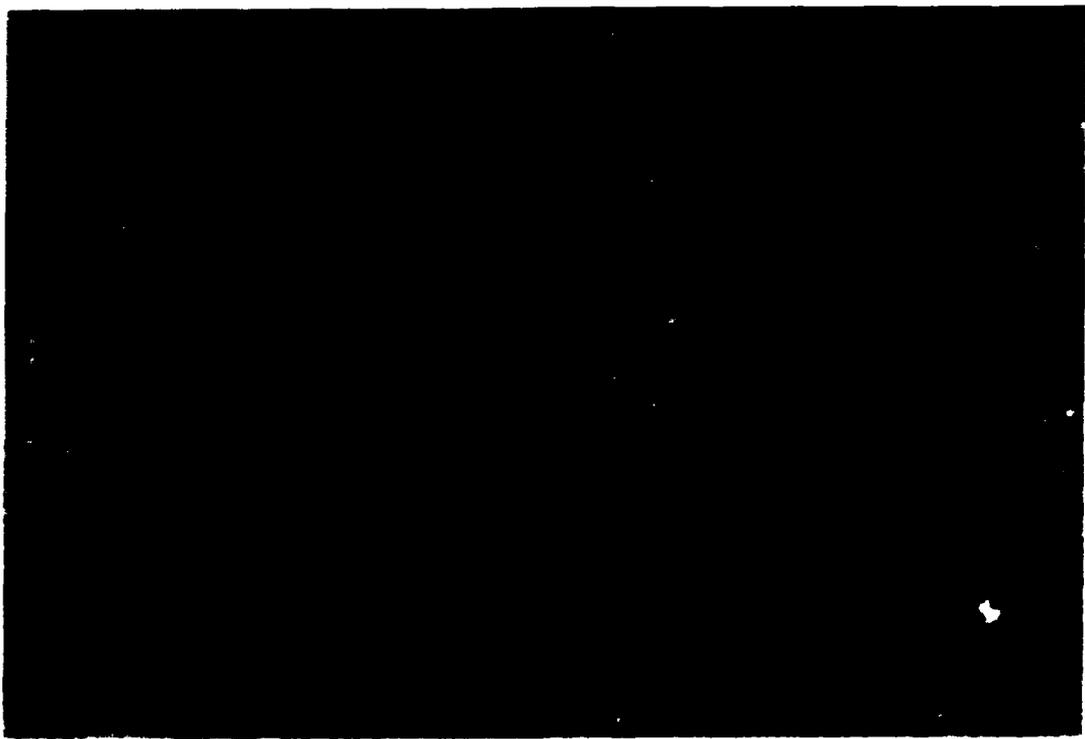
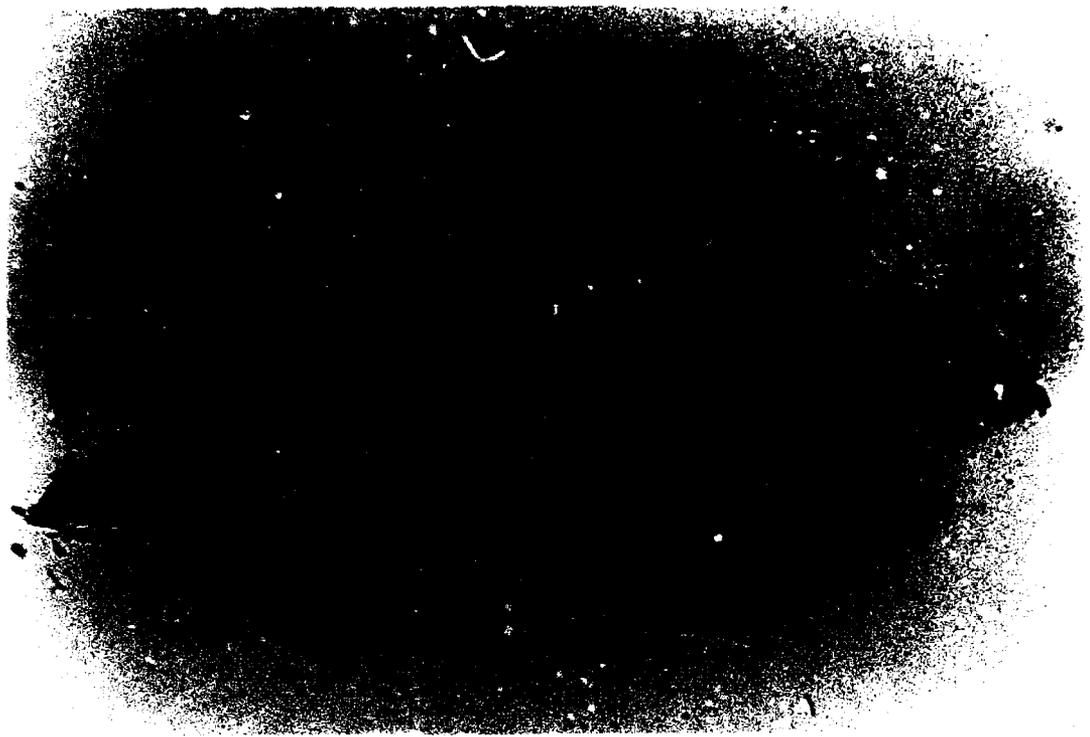
at 5 to 7 micrometers thickness, and stained according to Wolboch's geimsa modification.

Only one example each of a one exposure control biopsy and a two exposure control biopsy are shown as they were histologically similar in both immunization protocols. Figure 7 is a color print showing a cross section of an A. americanum larvae attached to an one exposure control guinea pig ear (2431). This section shows slight epidermal thickening around the attachment site with a limited infiltration of eosinophils located mainly in the dermis. An enlargement of this photomicrograph is seen in figure 8. The tick mouth parts, penetrating into the dermis, can clearly be seen surrounded by pink attachment cement. Characteristic of this tick genus, there is little or no cement on the epidermal surface, but it has extensively diffused into the host tissues. Eosinophils can be seen in the feeding lesion beneath the mouth parts and in the tick gut. Figure 9 shows a higher power view of this same feeding lesion and the mouth parts and cement are shown at the right side of the photograph. The eosinophils can be seen degranulating in the feeding lesion. Approximately 100 micrometers from the feeding lesion, in one exposure controls, the eosinophils are seen in lower concentration and numerous degranulating mast cells are also seen, as depicted in figure 10.

A typical reaction to tick feeding in a two exposure control, expressing acquired resistance, is seen in figure 11. One sees a highly thickened epidermis, intense basophilic cellular infiltration with the formation of an epidermal vesicle or bulla. Figure 12 is a higher power view of the vesicle shown in figure 11. Some eosinophils

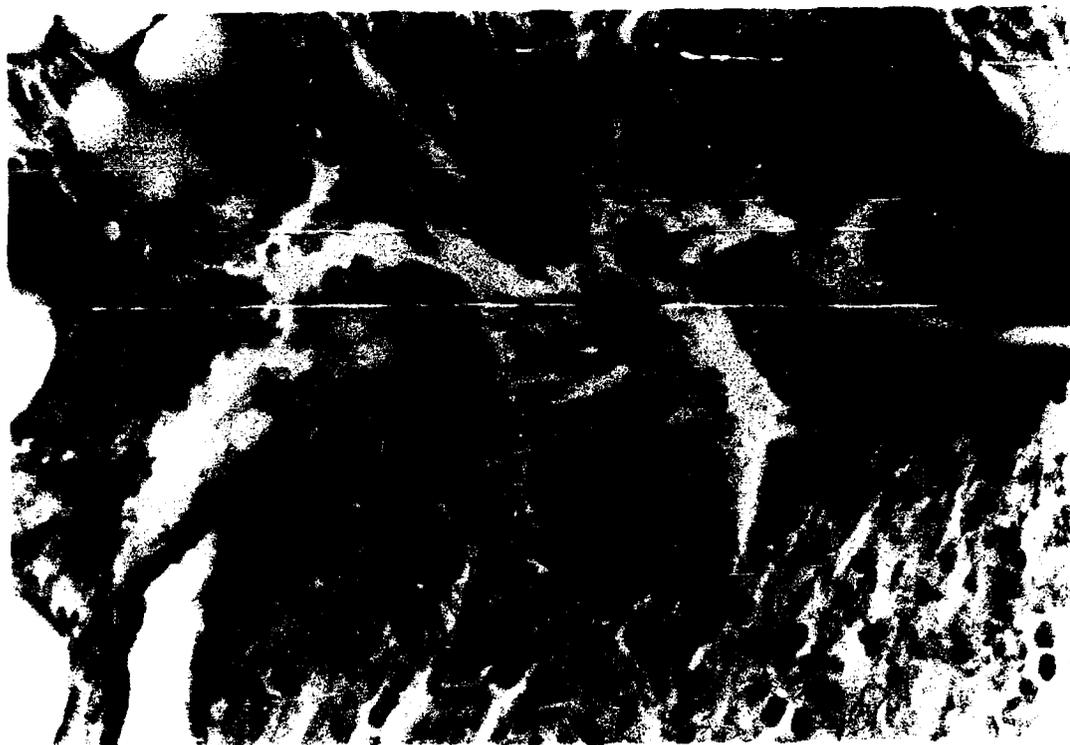
**Figure 7.** Cross section of Amblyomma americanum larva attached to the ear of an one exposure control guinea pig. This section shows slight thickening of the epidermis (T) around the attachment site with a limited to moderate infiltration of eosinophils. (original magnification: 5 x)

**Figure 8.** An enlargement of figure 7 showing the tick mouth parts (MP), which are penetrating into the dermis, surrounded by pink attachment cement (A). Characteristic of this tick genus there is little or no cement on the epidermal surface, but it has diffused into host tissues. Eosinophils (E) can be seen in the feeding lesion and eosinophil granules (G) are seen in the tick gut. (original magnification: 50 x)



**Figure 9.** View of the feeding lesion beneath the mouth parts of Amblyomma americanum larva pictured in figure 7. The mouth parts (MP) and attachment cement (A) are shown to the right. The eosinophils nearest the cement are degranulating (D) and the released granules (G) are seen in the surrounding area. (original magnification: 50 x)

**Figure 10.** Approximately 100 micrometers from the tick feeding lesion, on an one exposure animal, eosinophils (E) are seen in lower concentration than at the feeding lesion. Numerous, dark blue edged, irregularly shaped, degranulating mast cells (M) are also observed. (original magnification: 125 x)



**Figure 11.** A typical reaction to tick feeding in a two exposure control, expressing acquired resistance. Highly thickened epidermis (T), intense basophilic cellular infiltration (B) and intraepidermal vesicles (V) are characteristic histologic findings at tick attachment sites on resistant guinea pigs. (original magnification: 12.5 x)

**Figure 12.** A higher power view of the intraepidermal vesicle shown in figure 11. Majority of the infiltrating cells found in this vesicle appear to be basophilic and mononuclear (MN), while some eosiniphils(E) and neutrophils (N) can also be identified. (original magnification: 125 x)



and neutrophils can be identified, but the majority of the infiltrate in the vesicle appear to be basophilic and mononuclear. Attachment cement can be seen in this vesicle which appears close to being sloughed from the epidermal surface.

Guinea pigs immunized with A. americanum whole ova extract had reactions at tick attachment sites which were histologically more like the resistant two exposure controls than those observed in the one exposure control. This reaction can be seen in figure 13 and it is characterized by prominently thickened epidermis around the attachment site and a moderate, mixed, cellular infiltrate consisting of eosinophils, neutrophils, and lymphocytes. A rather intense cellular infiltrate invading the epidermis around the attachment site is of mixed cellular types as shown by figure 14. This higher power view of the left edge of the attachment site contains easily identified eosinophils, neutrophils, and a lymphocyte.

Guinea pig 2387, from immunization protocol I, was immunized with the  $\geq 100$  Kdal ultrafiltration fraction of A. americanum ova extract. The cutaneous reaction of this animal at a tick attachment site can be seen in figures 15 and 16. An intraepidermal vesicle, containing a moderate infiltrate of eosinophils and lymphocytes, is present. The predominant cell type appears to be the eosinophil, but a significant lymphocyte component is also present.

The guinea pigs from protocol I, immunized with the 30-100 Kdal ultrafiltration fraction, had very intense basophilic cellular infiltrate, formation of epidermal vesicles, and highly thickened epidermis around the tick attachment sites, one of which is demonstrated in

**Figure 13.** Cellular response at tick attachment site on a guinea pig immunized with ten micrograms of Amblyomma americanum whole ova extract per 250 g body weight. Note the thickened epidermis (T) around the attachment site and the moderate mixed cellular infiltrate consisting of eosinophils, neutrophils, and lymphocytes. (original magnification: 50 x)

**Figure 14.** The left edge of the feeding lesion seen in figure 13 contains a mixed cellular response consisting largely of eosinophils (E), a few neutrophils (N), and lymphocytes (L). (original magnification: 125 x)



**Figure 15.** Cutaneous reaction at tick attachment site on guinea pig immunized with 7.5 micrograms of  $\geq 100$  Kdal fraction of Amblyomma americanum ova extract. An intraepidermal vesicle (V), still communicating with the dermis and containing a moderate infiltrate of eosinophils and lymphocytes, is present.

(original magnification: 50 x)

**Figure 16.** Vesicle at tick attachment site with moderate infiltrate consisting of eosinophils (E) and lymphocytes (L). In this higher power view of the tick attachment site shown in figure 15, it is clear that the dominant cell type is the eosinophil, but, a significant lymphocyte component is also present. (original magnification: 125 x)

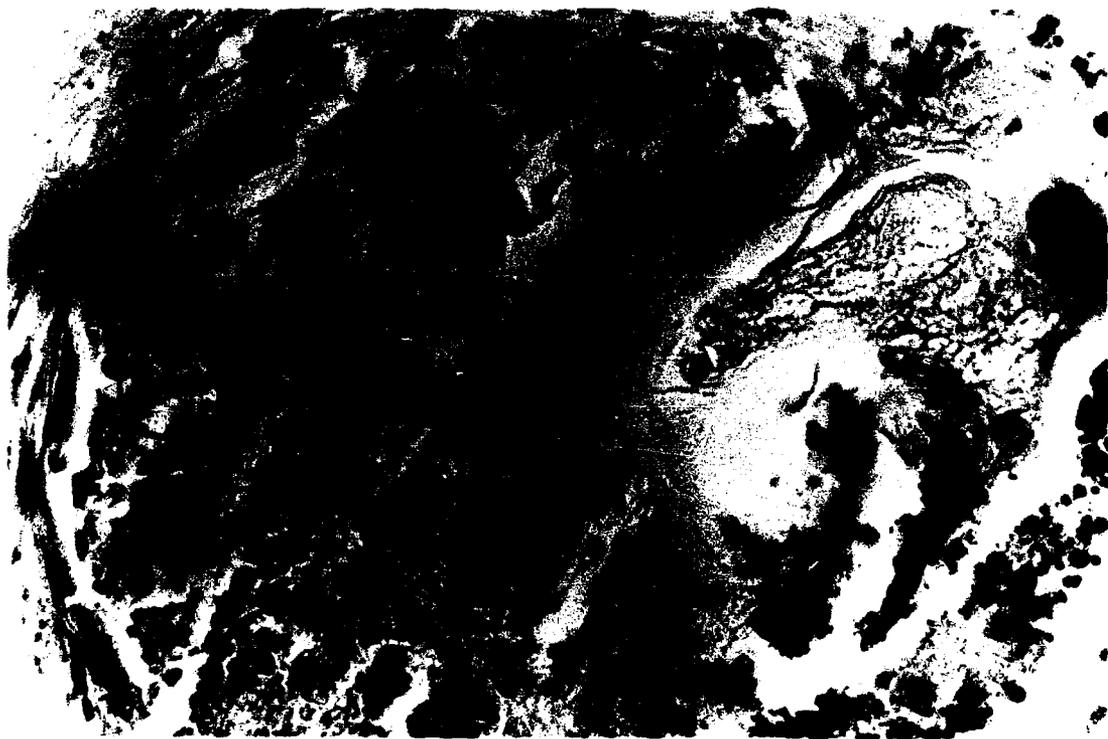
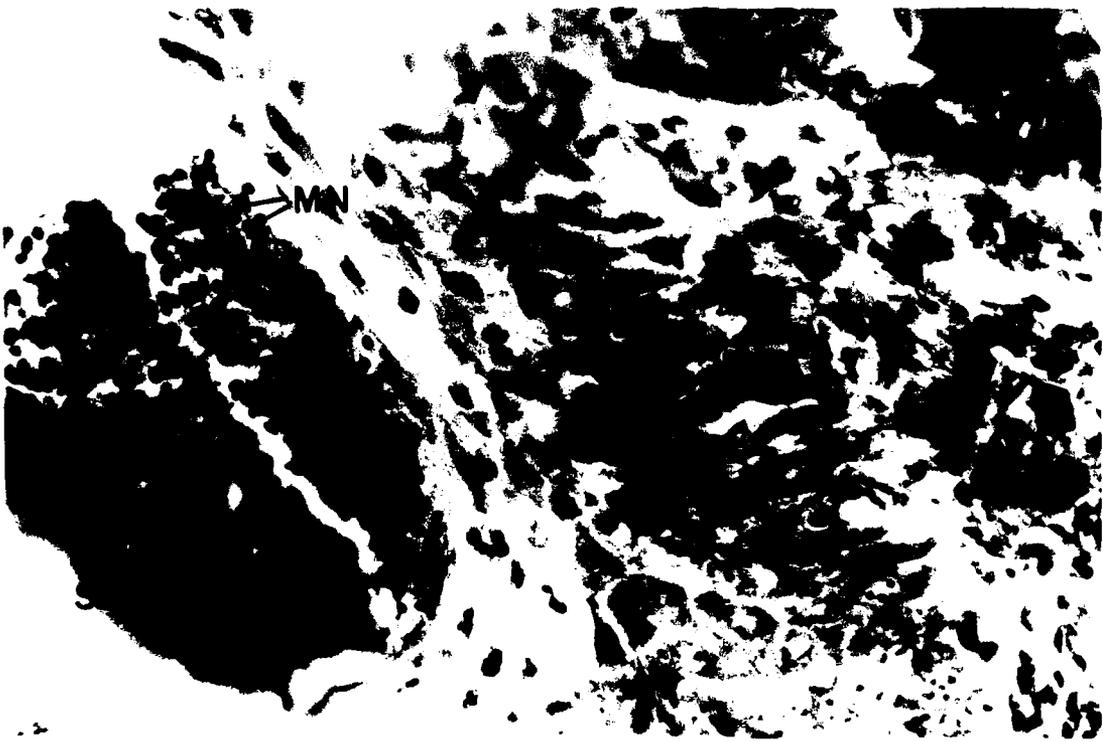


figure 17. At five days the vesicle is nearly ready to be sloughed from the epidermal surface. A higher power view of the vesicle pictured in figure 17 is shown in figure 18 and reveals that most cells have degenerated, leaving both segmented and mononuclear nuclei. The mononuclear cells appear to have been mainly lymphocytes, while the segmented cells were probably eosinophils and neutrophils, but could have included basophils. Numerous eosinophils and basophilic cells can be seen infiltrating the surrounding tissue.

Sephadex G-200 peak 1 immunized guinea pigs responded to larval infestation with a slight infiltration of eosinophils at the tick attachment site, which is shown in figure 19. A higher power view of this feeding lesion and attachment cement are shown in figure 20. The eosinophils are releasing their granules into the surrounding area. Very few cells infiltrated the epidermis near the sides of the pink attachment cement and those present were predominantly eosinophils. Immunization with Sephadex G-200 peak 2 resulted in a histologic picture very similar to that seen in one exposure controls and that seen in animals immunized with peak 1, both of which had very slight infiltrations of eosinophils at tick attachment sites. The partial outline of cuticle and muscle of a larval tick and pink attachment cement in host tissue dominate figure 21. A moderate infiltration of eosinophils characterizes the response of this guinea pig, immunized with Sephadex G-200 peak 2 of A. americanum ova extract. Guinea pigs immunized with Sephadex G-200 peak 3, had reactions to tick attachment like the one seen in figure 22, with a higher power view shown in figure 23. This reaction is similar to those observed in animals

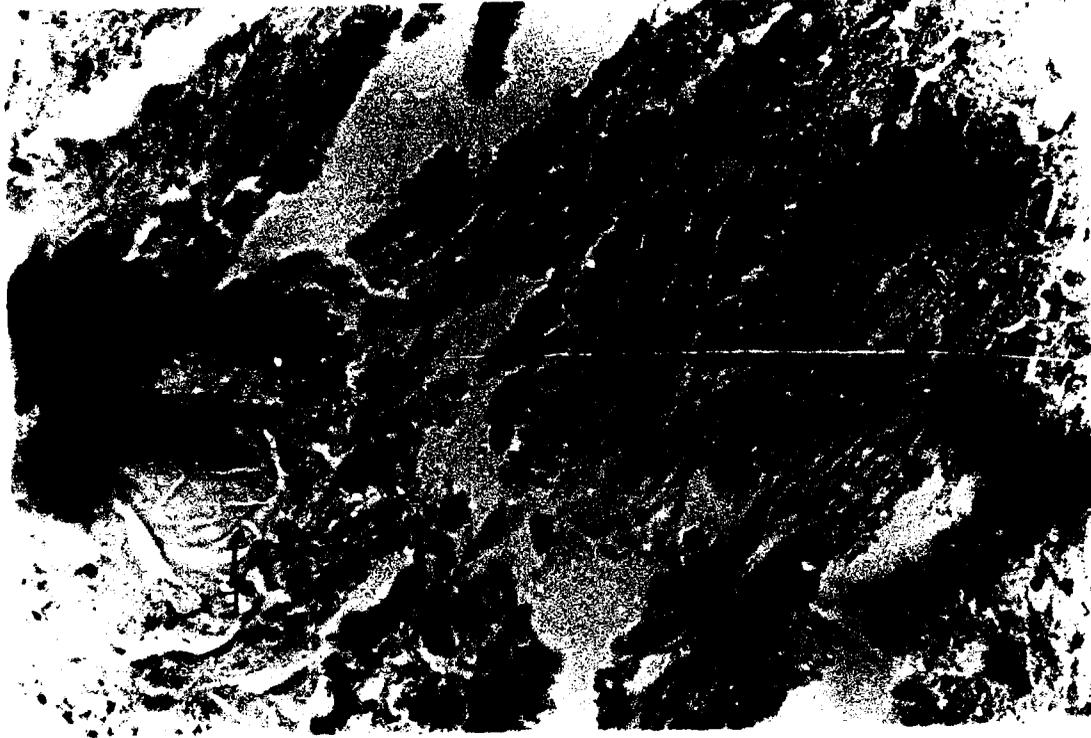
**Figure 17.** Intense basophilic response occurring at tick attachment site on a guinea pig immunized with five micrograms of a 30-100 Kdal fraction of Amblyomma americanum ova extract. A vesicle (V) formed at the tick attachment site is close to being sloughed from the epidermal surface. (original magnification: 50 x)

**Figure 18.** A higher power view of the vesicle shown in figure 17 reveals, that most cells have degenerated leaving both segmented (SN) and mononuclear (MN) nuclei. Numerous eosinophils and basophilic cells can be seen in the surrounding tissues. (original magnification: 125 x)



**Figure 19.** The cellular response at a tick attachment site on a guinea pig immunized with Sephadex G-200 peak 1. A slight infiltration of eosinophils can be seen in the vicinity of the tick attachment cement (A). (original magnification: 50 x)

**Figure 20.** A higher power view of the feeding lesion and attachment cement shown in figure 19. The eosinophils near the cement are releasing their granules (G) into the surrounding area. Very few cells infiltrated the epidermis near the sides of the cement, and those present were predominantly eosinophils (E). (original magnification: 125 x)



**Figure 21.** The partial outline (O) of cuticle and muscle of a tick and pink attachment cement (A) dominate this photomicrograph. A moderate infiltration of eosinophils (E) characterizes the response of this guinea pig, immunized with Sephadex G-200 peak 2 of Amblyomma americanum ova extract, to tick infestation. This animal displayed no more resistance than a one exposure control guinea pig. (original magnification: 12.5 x)

**Figure 22.** Guinea pigs, immunized with Sephadex G-200 peak 3, mount a cellular response, characterized by eosinophilia, at tick attachment sites. Similar to the reactions found in animals immunized with Sephadex G-200 peaks 1 and 2, this reaction is also dominated by the presence of eosinophils (E). The degree of eosinophil infiltration is greater than that seen in animals immunized with Sephadex G-200 peaks 1 and 2, and includes invasion of the epidermis around the attachment cement (A) resulting in a greatly thickened epidermis (T) at the attachment site. (original magnification: 12.5 x)



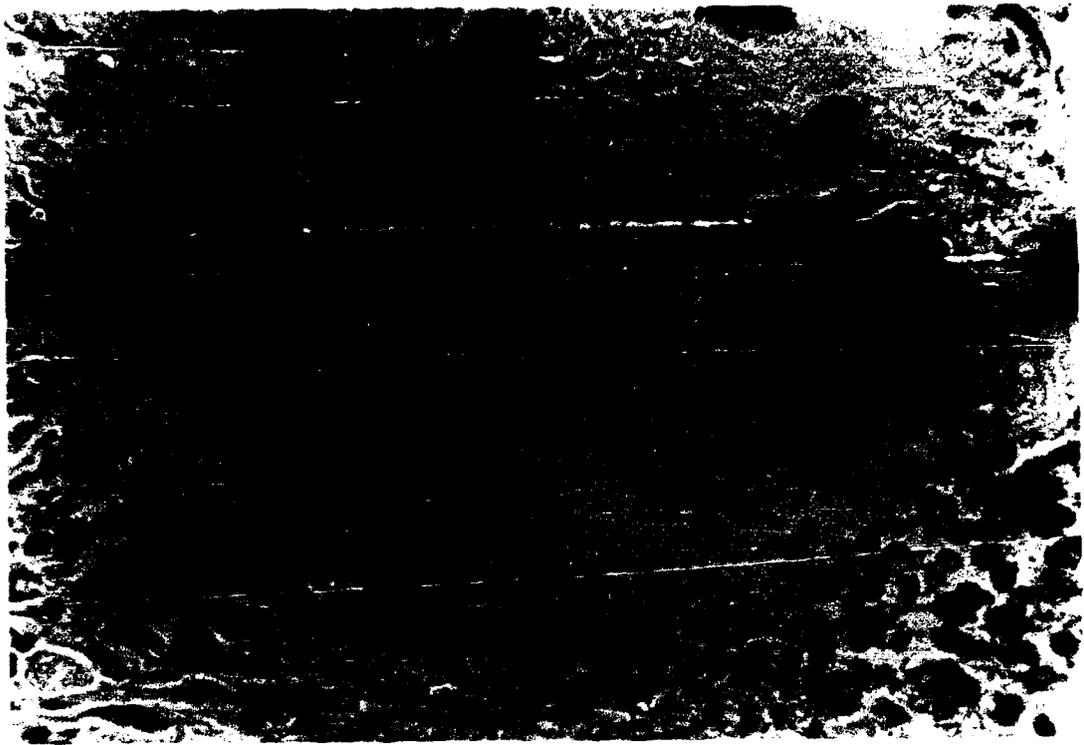
immunized with Sephadex G-200 peaks 1 and 2 in that the reaction is dominated by the presence of eosinophils. Although similar, the eosinophil infiltration is more intense than that seen in animals immunized with Sephadex G-200 peaks 1 and 2, and includes eosinophils invading the epidermis around the tick attachment cement. This influx of cells has resulted in a greatly thickened epidermis surrounding the attachment site. What appear to be degranulating mast cells were observed a short distance from the tick attachment site in peaks 1, 2, and 3 immunized animals. Similar observations were noted in one exposure controls approximately 100 micrometers from tick attachment sites.

A distinct attachment site was not found in the sections obtained from PBS immunized controls from immunization protocol II. Only a slight infiltrate, consisting of eosinophils and accumulation of collagen along with numerous degranulating mast cells, was observed and is pictured in figure 24.

Infiltrating white blood cells were identified and counted in the area of three oil immersion fields, ten micrometers from the attachment site or cement for each biopsy in immunization protocols I and II. Thus with pairs of guinea pigs biopsied, a mean and standard deviation (SD) were calculated for each cell type observed in six oil immersion fields. These data are listed in table 10 for protocol I and table 11 for protocol II. The greatest difference observed between cell types infiltrating tick attachment sites in protocol I and II is in the number of basophilic, or lymphoid, cells observed.

**Figure 23.** Eosinophils (D) close to attachment cement show degranulation. Guinea pigs immunized with Sephadex G-200 peak 3, responded to tick attachment with a more intense eosinophilia than guinea pigs immunized with peaks 1 and 2. Eosinophil infiltrate completely surrounds attachment cement (A). (original magnification: 125 x)

**Figure 24.** Response to tick infestation observed in the PBS immunized control. The distance of this section from the tick feeding site is not known, since the precise location of attachment was not found. The reaction consisted of a few eosinophils (E) and collagen (C), with numerous degranulating mast cells (M). This reaction is consistent with the findings observed in one exposure controls, approximately 100 micrometers from tick attachment sites. (original magnification: 125 x)



**Table 10. Cellular infiltration at tick attachment sites of guinea pigs from immunization protocol I.**

Immunization or treatment	Mean $\pm$ SD of cell types in six oil immersion fields				
	Eosinophils	Neutrophils	Lymphoid cells	Basophils	Mast cells
Whole ova extract	25.8 $\pm$ 16.2	0	9.1 $\pm$ 4.9	0	0.7 $\pm$ 0.8
$\geq$ 100 Kdal fraction	5.0 $\pm$ 4.9	0.5 $\pm$ 0.8	9.0 $\pm$ 6.0	0	1.5 $\pm$ 1.9
30 to 100 Kdal fraction	20.3 $\pm$ 10.8	0.7 $\pm$ 1.0	16.0 $\pm$ 9.0	0.3 $\pm$ 0.8	1.3 $\pm$ 1.5
Two exposure control	17.5 $\pm$ 9.7	1.0 $\pm$ 1.7	7.6 $\pm$ 8.2	0.2 $\pm$ 0.4	1.2 $\pm$ 1.6
One exposure control	3.3 $\pm$ 4.4	0.8 $\pm$ 1.6	8.3 $\pm$ 10.0	0	0.2 $\pm$ 0.4

Note:

One biopsy was taken from each animal.

Cells were identified and counted in the area of three oil immersion fields a standard distance of ten micrometers from the tick attachment site. The number of cells listed is the mean  $\pm$  the standard deviation (SD) for the cells counted in both biopsies.

Lymphoid cells include all basophilic, mononuclear cells which appear to be either lymphocytes or plasmacytes.

**Table 11. Cellular infiltration at tick attachment sites of guinea pigs from protocol II.**

Immunization or treatment	Mean $\pm$ SD of cell types in six oil immersion fields				
	Eosinophils	Neutrophils	Lymphoid cells	Basophils	Mast cells
Whole ova extract	10.7 $\pm$ 5.7	2.8 $\pm$ 1.5	3.2 $\pm$ 2.3	0	0.3 $\pm$ 0.8
Peak 1	22.3 $\pm$ 10.1	3.2 $\pm$ 1.9	0.5 $\pm$ 0.5	0.3 $\pm$ 0.8	1.3 $\pm$ 1.5
Peak 2	27.7 $\pm$ 13.0	1.5 $\pm$ 1.2	1.0 $\pm$ 1.3	0	1.3 $\pm$ 1.5
Peak 3	9.3 $\pm$ 10.3	1.7 $\pm$ 1.6	0.5 $\pm$ 1.2	0	0
PBS control	1.3 $\pm$ 1.2	0.2 $\pm$ 0.4	0.7 $\pm$ 1.2	0.2 $\pm$ 0.4	1.7 $\pm$ 2.1
Two exposure control	18.3 $\pm$ 8.7	0.5 $\pm$ 1.2	18.7 $\pm$ 19.7	0	0.3 $\pm$ 0.5
One exposure control	16.0 $\pm$ 5.4	1.2 $\pm$ 1.2	1.5 $\pm$ 1.8	0	1.7 $\pm$ 1.6

Note:

Cells were identified and counted in the area of three oil immersion fields a standard distance of ten micrometers from the tick attachment site. The number of cells listed is the mean  $\pm$  the standard deviation (SD) for the cells counted in both biopsies.

Lymphoid cells include all basophilic, mononuclear cells which appear to be either lymphocytes or plasmacytes.

The treatment of the controls for both immunization protocols was the same. Therefore, the control groups from immunization protocols I and II were combined for use in statistical analysis. Three one exposure control sections were combined. One section, on which no attachment site, and practically no cellular response were noted was not included. The mean number of cells in nine oil immersion fields was used as the value for the one exposure control group. The two exposure controls were also combined, giving the mean number of cells in 12 oil immersion fields as the two exposure control value. Multiple linear regression compared the mean values of each treatment group, first to the mean value for one exposure controls and then to the mean value for two exposure controls.

The mean number of eosinophils found in six oil immersion fields for pairs of guinea pigs from immunization protocol I ranged from 3.3, for the one exposure control, to 25.8, for animals immunized with whole ova extract. The mean number of lymphoid cells (lymphocytes and plasmacytes) ranged from 7.6, for the two exposure control, to 16.0 for the animals immunized with the 30-100 Kdal fraction of A. americanum whole ova extract. The mean numbers of lymphocytes is quite similar for all groups except for the 30-100 Kdal fraction immunized group which has nearly double the number found in other groups. It must be pointed out that both the two exposure controls and the 30-100 Kdal fraction immunized animals had developed vesicles which contained very high concentrations of lymphocytes. It is possible that migration into the vesicle may have decreased lymphocyte numbers in the area counted. Findings are dependent on the time of sampling. The number of eosino-

phils observed appears to be relative to the degree of resistance observed in this immunization protocol. The largest numbers of eosinophils and the highest levels of resistance were observed in guinea pigs immunized with whole ova extract, 30-100 Kdal fraction, and the two exposure controls.

The mean numbers of eosinophils in immunization protocol II ranged from 1.3, for the PBS control, to 27.7, for the peak 2 immunized group. Although the peak 2 immunized group, which had the greatest apparent resistance as determined by ability to molt from larvae to nymphs, also had the greatest number of eosinophils, the relationship of the mean number of eosinophils to resistance does not seem to be as direct as it was for immunization protocol I. This is reflected by the fact that the one and two exposure controls had nearly the same mean number of eosinophils and the widest differences in expressed resistance. Lymphocytes were noted in significant numbers only in the two exposure controls, with a mean of 18.7. The number of lymphocytes in the whole ova extract immunized group, with a mean of 3.2, had the next highest number of lymphocytes.

Attempting to compare cell types and resistance within each of these immunization protocols results in one possible trend being noted: animals with greater levels of resistance seem, in general to have higher numbers of eosinophils and lymphoid cells present. There are contradictions to this generalization in data from this histology.

Statistical analysis, of cell types observed in tissue sections, showed that the PBS controls, which had less resistance than any other group, had significantly fewer eosinophils than the one exposure

controls (0.05 level) and fewer than the two exposure controls (0.01 level). Peak 2 immunized animals, having a high level of resistance, had significantly more eosinophils than the one exposure controls (0.053 level). The whole ova extract immunized group from immunization protocol I, but not from immunization protocol II, had significantly more eosinophils than the one exposure control (0.014 level). The comparison of lymphocytes showed peak 1, peak 2, peak 3, and PBS immunized groups had significantly fewer lymphoid cells than either one exposure controls (0.05 level) or two exposure controls (0.01 level). The whole ova immunized group from immunization protocol II had significantly fewer lymphoid cells than two exposure controls (0.015 level), while the 30-100 Kdal fraction immunized group was the only group which had more lymphoid cells than two exposure controls (0.054 level).

In addition to the variation observed in cellular response between treatment groups in each immunization protocol, a notable variation existed between pairs of animals in each group, demonstrated by large standard deviations for cell types. Comparing the two immunization protocols to each other, a difference can be seen in the numbers of eosinophils and lymphoid cells observed in animals treated similarly, such as whole ova extract immunized groups and control groups. In general immunization protocol II animals had fewer lymphoid cells. This may indicate a difference in the stimuli of different challenging tick groups or variation in genetically regulated host response mechanisms.

## Dot-ELISA Titers

Serum, collected from animals immunized in protocols I and III, was titered by the Dot-ELISA method. Sera from immunization protocol I animals were titered against A. americanum ova extract while sera from protocol III animals were titered separately against both A. americanum ova extract and A. americanum gut extract. The titers are listed in tables 12 and 13. The antibody titers of guinea pigs from immunization protocol I to Amblyomma americanum were uniformly high in all immunized, and then challenged animals. Using the rule of thumb that plus or minus one dilution is acceptable within procedure variation, there is little difference between the treatment groups. Using the closest values 1:32,768; 1:8,192; and 1:16,384 the maximum difference is two dilutions; while using the most extreme values 1:65,536 and 1:4,096 there is a four dilution variation. The titer does not seem to relate directly to resistance as guinea pigs immunized with 30-100 Kdal fraction were the most resistant group but had only mid range titers, 1:16,384, for these three groups.

Guinea pigs from immunization protocol III were immunized with 20 micrograms of gut antigen in addition to 20 micrograms of either A. americanum whole ova extract or Sephadex G-200 peaks 1,2, or 3 per 250 g body weight. Their antibody titers which ranged from 1:512 to 1:8192 to A. americanum ova extract and 1:128 to 1:4096 to A. americanum gut extract, seem to be lower, in general, than the titers of animals from immunization protocol I that were immunized with only one extract. The control guinea pigs immunized with gut only appear to demonstrate that

**Table 12. The antibody titer of guinea pigs from immunization protocol I to Amblyomma americanum ova extract.**

Guinea pig #	Immunization	Titer
2385	whole ova extract	1:32,768
2386	whole ova extract	1:65,536
2387	≥ 100 Kdal fraction	1:8,192
2388	≥ 100 Kdal fraction	1:4,096
2389	30-100 Kdal fraction	1:16,384
2390	30-100 Kdal fraction	1:16,384

**Note:**

Antigen was approximately 100 ng of Amblyomma americanum ova extract per disk.

First antibody, guinea pig serum, diluted from 1:2 to 1:524, 288 using two-fold dilutions.

Second antibody was rabbit anti-guinea pig IgG-HRPO diluted 1:200. Maximum reaction time with substrate was 15 minutes.

**Table 13. The antibody titer of guinea pigs from immunization protocol III to Amblyomma americanum ova extract and gut extract.**

Guinea pig #	Immunization	Titer to	
		ova extract	gut extract
2439	whole ova extract plus gut	1:8,192	1:256
2440	whole ova extract plus gut	1:4,096	1:128
2441	Peak 1 plus gut	1:4,096	1: 128
2442	Peak1 plus gut	1:8,192	1:1,024
2443	Peak 2 plus gut	1:4,096	1:1,024
2444	Peak 2 plus gut	1:4,096	1:4,096
2445	Peak 3 plus gut	1:512	1:1,024
2446	Peak 3 plus gut	1:8,192	1:4,096
2447	Gut only	1:512	1:1,024
2448	Gut only	1:1,024	1:1,024

**Note:**

Antigen was approximately 100 ng of Amblyomma americanum ova extract or Amblyomma americanum gut extract per disk.

First antibody, guinea pig serum, was diluted two-fold from 1 :2 to 1:65,536.

Second antibody was rabbit anti-guinea pig IgG-HRPO diluted 1:200. Maximum reaction time with substrate was 15 minutes.

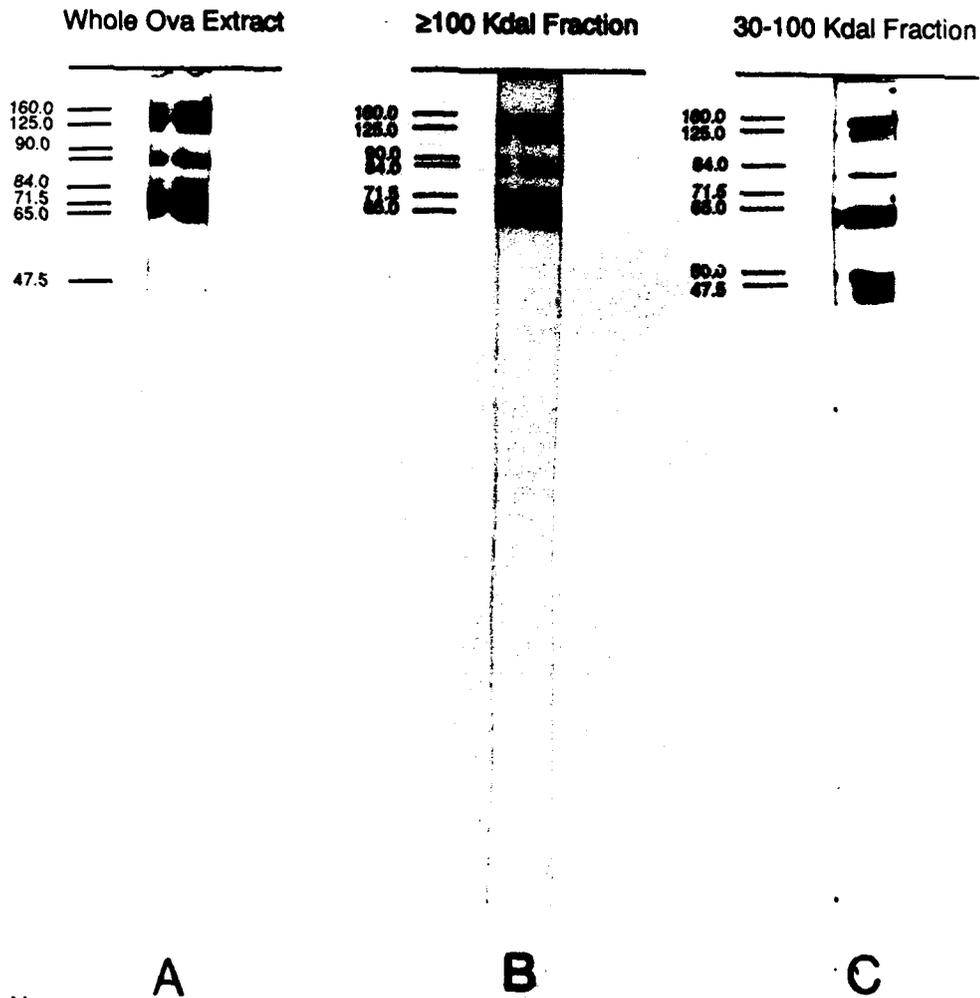
there may be shared, or similar, epitopes in A. americanum ova and gut extracts because they have the same titer to both antigens. It is possible that these titers and reactivity, to epitopes other than gut epitopes, are a result of the larval challenge.

#### Immunoblotting

Serum was collected from immunized and challenged guinea pigs used in protocols I, II, III, and IV after tick infestation. The antigen preparation used in each protocol, except IV, were fractionated by SDS-PAGE and electroeluted to nitrocellulose. For protocol IV, 27,000 x g supernatant was fractionated by SDS-PAGE and then electroeluted to nitrocellulose for use in immunoblot analysis instead of whole gut extract. Dot-ELISA trials showed that antibodies in the serum of these animals recognized epitopes in 27,000 x g supernatant as strongly as those in whole gut extract were recognized. The serum was used as first antibody in immunoblotting. Antibody in the serum immunolocalized or bound to polypeptide bands it recognized in the immobilized antigen. Immunodetection involved use of rabbit anti-guinea pig IgG conjugated with horseradish peroxidase (HRPO) and a precipitable substrate. The resulting bands, as seen in figure 25, were measured for distance of migration and relative mobility factors (Rf) were calculated. The approximate molecular weights were then read from a graph depicting the Rf of the standard molecular weight markers.

Figure 25 depicts the results of immunoblot analysis performed using A. americanum whole ova extract electrophoresed under reducing

**Figure 25. Immunoblot analysis of sera from guinea pigs immunized with *Amblyomma americanum* whole ova extract or defined molecular weight range ultrafiltration fractions of ova extract and then challenged with 100 *Amblyomma americanum* larvae.**



Note:

First antibody, guinea pig serum, diluted 1:500; second antibody, rabbit anti-guinea pig IgG-HRPO diluted 1:200.  
*Amblyomma americanum* ova extract, loaded at six micrograms of protein per lane, electrophoresed under reducing conditions on a 12% separating gel at 30 milliamperes until the running front reached 11.5 cm, then electroeluted to nitrocellulose strips at 150 milliamperes for 18 hours.

conditons and then electroeluted to nitrocellulose as antigen and serum collected from the guinea pigs immunized in immunization protocol I post tick challenge as first antibody. (See Materials and Methods for immunization details.) The serum from a guinea pig immunized with A. americanum whole ova extract recognized polypeptide bands with molecular weights ranging from 47.5 to 160 Kdal. The guinea pig immunized with  $\geq 100$  Kdal fraction of A. americanum whole ova extract had serum that recognized polypeptide bands with molecular weights ranging from 65 to 160 Kdal. Serum from the guinea pig immunized with the 30-100 Kdal fraction of A. americanum whole ova extract recognized polypeptide bands ranging from 47.5 to 160 Kdal in molecular weight. It is interesting to note that heavy molecular weight polypeptide bands have been recognized more intensely on nitrocellulose strip A than on nitrocellulose strip C. While light molecular weight bands have been recognized more intensely on strip C than on strip A. This is probably a direct reflection of immune response to the concentration of those molecular weight moieties with which the guinea pigs were immunized. Of course it is possible that the challenging tick infestation may have selectively stimulated an increased reactivity to some of the polypeptide bands recognized.

The number and molecular weights of polypeptide bands recognized by serum from each immunized animal are listed in tables 14, 15, 16, 17, and 18 for immunization protocol I, II, III ova, III gut, and IV, respectively. Serum collected from the guinea pigs immunized in immunization protocol I recognized polypeptide bands, in A. americanum whole ova extract electrophoresed under reducing conditions and

**Table 14. The molecular weights of polypeptide bands in Amblyomma americanum ova extract recognized by sera of guinea pigs from immunization protocol I.**

Guinea pig #	Treatment group	# of bands recognized	Approximate molecular weight in Kdal
2385	Whole ova extract	4	185.0, 152.5, 111.0, 82.0
2386	Whole ova extract	6	185.0, 152.5, 111.0, 89.0, 79.0, 73.0
2387	≥ 100 Kdal fraction	6	185.0, 152.5, 111.0, 92.5, 82.0, 79.0
2388	≥ 100 Kdal fraction	5	185.0, 152.0, 111.0, 82.0, 79.0
2389	30-100 Kdal fraction	7	185.0, 165.0, 111.0, 82.0, 79.0, 60.0, 56.5
2390	30-100 Kdal fraction	8	185.0, 152.5, 111.0, 92.5, 82.0, 79.0, 58.0, 55.0

**Note:**

Normal guinea pig serum recognized no bands in this Amblyomma americanum whole ova extract. Approximately ten micrograms of Amblyomma americanum ova extract was loaded per lane on SDS-PAGE gel. This was electrophoresed under reducing conditions at 30 milliamperes until the running front reached 11.5 cm. Then it was electroeluted to nitrocellulose at 150 milliamperes for 18 hours.

Guinea pig sera, diluted 1:32-1:256, was used as the first antibody; the second antibody was rabbit anti-guinea pig IgG-HRPO diluted 1:200. Maximum reaction time with the substrate was 30 minutes.

**Table 15. The molecular weights of polypeptide bands in Amblyomma americanum ova extract recognized by sera of guinea pigs from immunization protocol II.**

Guinea pig #	Treatment group	# of bands recognized	Approximate molecular weight in Kdal
2417	Whole ova extract	20	245.0, 225.0, 220.0, 215.0, 200.0, 185.0, 145.0, 125.0, 116.2, 92.5, 82.0, 63.0, 55.0, 51.0, 49.7, 46.0, 44.0, 40.5, 38.5, 25.5
2419	Whole ova extract	12	257.0, 225.0, 200.0, 152.5, 145.0, 89.0, 81.0, 63.0, 58.0, 56.5, 52.5, 51.0
2420	Peak 1	8	200.0, 145.0, 89.0, 55.0, 51.0, 47.5, 46.0, 45.0
2421	Peak 2	7	200.0, 152.5, 145.0, 81.0, 56.5, 47.5, 44.0
2422	Peak 3	16	240.0, 225.0, 222.0, 215.0, 200.0, 152.5, 137.0, 92.5, 81.0, 63.0, 58.0, 56.5, 53.5, 52.5, 46.0
2432	PBS	2	51.0, 46.0

**Note:**

Normal guinea pig serum recognized no bands in this Amblyomma americanum whole ova extract. Approximately ten micrograms of Amblyomma americanum ova extract was loaded per lane on SDS-PAGE gel. This was electrophoresed under reducing conditions at 30 milliamperes until the running front reached 11.5 cm. Then it was electroeluted to nitrocellulose at 150 milliamperes for 18 hours. Guinea pig sera, diluted 1:32-1:256, was used as the first antibody; the second antibody was rabbit anti-guinea pig IgG-HRP0 diluted 1:200. Maximum reaction time with the substrate was 30 minutes.

**Table 16. The molecular weights of polypeptide bands in Amblyomma americanum ova extract recognized by sera of guinea pigs from immunization protocol III.**

Guinea pig #	Treatment group	# of bands recognized	Approximate molecular weight in Kdal
2439	Whole ova plus gut	13	163.0, 150.0, 133.0, 128.0, 103.0, 87.0, 77.0, 66.5, 61.5, 58.0, 54.5, 41.5, 38.0
2440	Whole ova plus gut	13	163.0, 155.0, 150.0, 133.0, 128.0, 102.5, 83.0, 75.0, 65.5, 61.5, 54.5, 41.0, 38.0
2441	Peak 1 plus gut	14	163.0, 157.0, 150.0, 133.0, 128.0, 102.5, 82.5, 75.0, 65.5, 61.5, 57.0, 54.0, 40.3, 37.5
2442	Peak 1 plus gut	13	163.0, 150.0, 133.0, 128.0, 102.5, 82.3, 75.0, 66.5, 57.0, 54.0, 52.0, 40.3, 37.5
2443	Peak 2 plus gut	10	163.0, 155.0, 150.0, 133.0, 103.0, 75.0, 63.0, 59.0, 53.0 47.0
2444	Peak 2 plus gut	17	165.0, 157.0, 155.0, 150.0, 134.0, 128.0, 124.0, 102.5, 80.0, 75.5, 63.0, 61.0, 59.0, 53.0, 47.0, 43.5, 39.9
2445	Peak 3 plus gut	12	163.0, 155.0, 150.0, 133.0, 102.5, 75.5, 68.0, 63.0, 58.0, 53.5, 47.0, 38.3
2446	Peak 3 plus gut	9	163.0, 150.0, 102.5, 75.5, 68.0, 63.0, 61.0, 59.0, 43.5
2447	Gut only	7	165.0, 133.0, 102.5, 69.0, 67.0, 63.0, 59.0
2448	Gut only	6	165.0, 150.0, 133.0, 102.5, 68.0, 59.0

**Note:**

Normal guinea pig serum reacted with two bands in this antigen preparation: 70.0 and 64.0 kilodaltons (Kdal). Approximately ten micrograms of Amblyomma americanum ova extract were loaded per lane on SDS-PAGE gel. This was electrophoresed under reducing conditions at 30 milliamperes until the running front reached 11.5 cm. Then it was electroeluted to nitrocellulose at 150 milliamperes for 18 hours. Guinea pig sera, diluted 1:32-1:256, was used as the first antibody; the second antibody was rabbit anti-guinea pig IgG-HRPO diluted 1:200. Maximum reaction time with the substrate was 30 minutes.

**Table 17. The molecular weights of polypeptide bands in Amblyomma americanum gut extract recognized by sera of guinea pigs from immunization protocol III.**

Guinea pig #	Treatment group	# of bands recognized	Approximate molecular weight in Kdal
2439	Whole ova plus gut	8	153.0, 118.0, 108.0, 85.0, 75.0, 68.0, 63.0, 53.0
2440	Whole ova plus gut	9	153.0, 115.0, 110.0, 106.0, 85.0, 68.0, 63.0, 58.0, 51.0
2441	Peak 1 plus gut	11	153.0, 150.0, 145.0, 140.0, 135.0, 118.0, 106.0, 85.0, 74.0, 68.5, 62.0, 51.0
2442	Peak 1 plus gut	13	164.0, 153.0, 150.0, 145.0, 143.0, 135.0, 118.0, 106.0, 85.0, 74.0, 68.5, 62.5, 51.0
2443	Peak 2 plus gut	12	153.0, 150.0, 140.0, 133.0, 127.0, 118.0, 106.0, 86.0, 74.0, 61.0, 58.0, 51.0
2444	Peak 2 plus gut	14	162.0, 153.0, 145.0, 143.0, 137.0, 115.0, 106.0, 85.0, 71.0, 63.0, 60.0, 55.5, 52.0, 51.0
2445	Peak 3 plus gut	8	153.0, 150.0, 144.0, 117.0, 106.0, 69.5, 62.5, 51.0
2446	Peak 3 plus gut	10	162.0, 145.0, 143.0, 127.0, 117.0, 113.0, 106.0, 81.0, 69.5, 62.5
2447	Gut only	13	162.0, 153.0, 147.0, 144.0, 135.0, 117.0, 110.0, 106.0, 86.0, 69.5, 63.0, 59.0, 50.0
2448	Gut only	16	162.0, 153.0, 147.0, 145.0, 136.0, 136.0, 117.0, 106.0, 91.0, 86.0, 77.0, 66.5, 60.0, 53.5, 42.0, 39.5

**Note:**

Normal guinea pig serum reacted with two bands in this antigen preparation: 66.5 and 60 kilodaltons (Kdal). Approximately ten micrograms of Amblyomma americanum gut extract were loaded per lane on SDS-PAGE gel. This was electrophoresed under reducing conditions at 30 milliamperes until the running front reached 11.5 cm. Then it was electroeluted to nitrocellulose at 150 milliamperes for 18 hours. Guinea pig sera, diluted 1:32-1:256, was used as the first antibody; the second antibody was rabbit anti-guinea pig IgG-HRPO diluted 1:200. Maximum reaction time with the substrate was 30 minutes.

**Table 18. The molecular weight of polypeptide bands in 27,000 x g supernatant recognized by sera of guinea pigs from immunization protocol IV.**

Guinea pig #	Treatment group	# of bands recognized	Approximate molecular weight in Kdal
2457	BBF	2	148.0, 60.4
2458	BBF	7	156.0, 148.0, 137.0, 133.0, 125.0, 113.0, 60.8
2459	27,000 x g	10	158.0, 153.0, 148.0, 144.0, 141.0, 113.0, 68.1, 66.2, 63.9, 60.8
2460	27,000 x g	2	148.0, 60.8
2462	PBS control	1	60.8

**Note:**

Normal guinea pig serum recognized one band at 60.8 kilodaltons (Kdal) in this Amblyomma americanum 27,000 x g supernatant.

Immobilized antigen: 27,000 x g supernatant fractionated on a 12% SDS-PAGE gel, run under reducing conditions at 30 milliamperes until the running front reached 11.5 cm, then electroeluted to nitrocellulose strips at 150 milliamperes for 18 hours.

First antibody: sera from guinea pigs immunized with either BBF or 27,000 x g supernatant, then infested with four pairs of adult Amblyomma americanum, diluted 1:16-1:32.

Second antibody: rabbit anti-guinea pig IgG-HRPO diluted 1:200.

Maximum reaction time with the substrate was 30 minutes.

electroeluted to nitrocellulose, ranging in molecular weight from approximately 55 to 185 Kdal. All six immunized animals recognized bands with molecular weights of 185 and 111 Kdal; while all but one animal recognized bands with molecular weights of 82 and 152 Kdal. Guinea pigs immunized with the 30-100 Kdal fraction of A. americanum whole ova extract uniquely recognized bands in the 55 to 58 Kdal range. This may be due to the high concentration of two polypeptide bands in the 30-100 Kdal fraction which had molecular weights of approximately 53.5 and 58 Kdal. Figure 4 illustrates that these two bands are present in the whole ova extract at a lower concentration but are totally absent in the  $\geq 100$  Kdal fraction.

Immunoblot analysis of serum, collected from guinea pigs immunized in protocol II and challenged with 100 A. americanum larvae, recognized polypeptide bands in A. americanum whole ova extract which ranged from 46 to 257 Kdal in molecular weight. Serum from multiple animals recognized bands with the same or similar molecular weights, but, only one band, a 200 Kdal band, was recognized by all animals immunized with antigens. Serum from the PBS immunized control recognized two bands, 51 and 46 Kdal, indicating nonspecific reactivity.

Serum from animals immunized in immunization protocol III recognized polypeptide bands with molecular weights ranging from 37.5 to 165 Kdal in the A. americanum ova extract and 39.5 to 153 Kdal in the A. americanum gut extract. Only one band in the ova extract, with a molecular weight of 150 Kdal, was recognized by the sera of all immunized animals. The diversity in both numbers and molecular weights of bands recognized, even between the two animals in each treatment

group, precludes defining specific patterns. As with the ova extract, some bands in gut extract, such as 51, 53, and 153 Kdal, are recognized by serum from a majority of animals, but no single band in this antigen was recognized by sera from all animals.

The sera, from all antigen immunized animals in immunization protocol IV, recognized two polypeptide bands in 27,000 x g supernatant with a molecular weights of approximately 60 Kdal and 148 Kdal. The 60 Kdal band was also recognized by the PBS control, indicating this was a nonspecific reaction.

Based on the lack of similarity between pairs of immunized animals in recognizing polypeptide bands, thus giving no clear cut patterns, it is not possible to correlate any particular molecular weight bands in any of these antigens with resistance. It is apparent that a significant variation in both the number of bands recognized and the molecular weights of bands recognized exists between pairs of animals as well as between different immunization protocols. This may reflect genetic variation in the immune response capabilities of these nonsyngeneic guinea pigs. Other possibilities include lack of homogeneity of the antigen preparation or variation in the concentration injected.

#### Monoclonal Antibodies

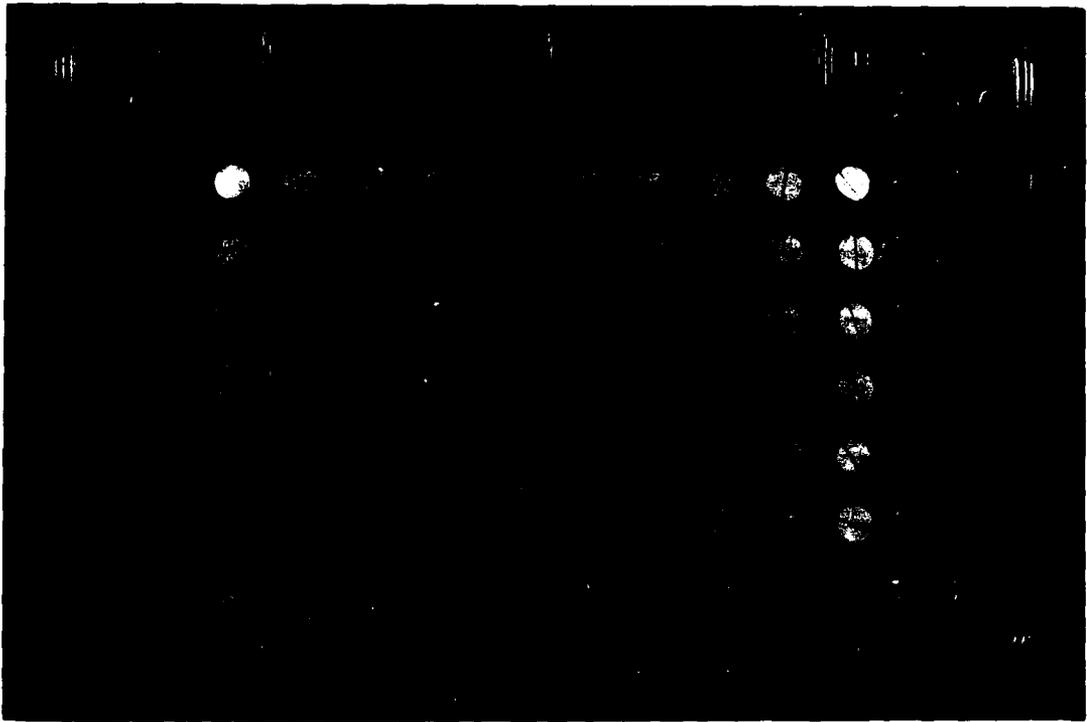
Mice were immunized according to the three regimens listed in the materials and methods section. Cells from immunized mice were fused with NS-1 myeloma cells for production of hybridomas producing mono-

clonal antibodies to D. andersoni antigens. The fusion using spleen cells from mice infested with D. andersoni nymphs failed to produce any clones secreting antibody to D. andersoni SGA or ova extract.

The fusion using spleen cells from mice injected with D. andersoni ova extract resulted in 14 wells which contained clones that gave positive Dot-ELISA tests (figure 26) when screened for antibody against D. andersoni ova extract. Six of these wells were positive 16 days post fusion and all 14 wells were positive by 22 days post fusion. Due to fungal contamination of this original plate, two wells were lost and nine of the wells were pooled and frozen without further work. The three wells, D7, D8, and D9, producing very reactive antibodies were split with half of their contents being frozen and half used for cloning. The cells to be frozen were not counted. The hybridoma cells being cloned were counted and diluted to give one cell per three wells which is standard for formal cloning. These cells were diluted in growth medium containing  $1 \times 10^6$  thymocytes/ml for use as feeder cells. One cloned well, D7, resulted in 21 positive wells out of 60 plated. A second well, D8, produced 41 positive wells out of 60 plated, and the third well, D9, produced no positive clones.

Three wells were formal cloned from these two plates containing positive wells. One formal clone produced 34 positive wells, the second 31, and the third only one. Prior to expanding, or freezing, any of these wells, either a break in sterile procedure or a contaminated vapor trap in the vacuum line led to contamination and all plates were lost to fungal contamination.

**Figure 26.** Dot-ELISA plate depicting screen for antibody production to Dermacentor andersoni ova extract by hybridoma cells resulting from immunization regimen I fusion. One hundred microliters of undiluted culture medium were used as the first antibody and the second antibody, diluted 1:400, was goat anti-mouse IgG tagged with horseradish peroxidase (HRPO). Horseradish peroxidase reacted with the precipitable substrate, 4-chloro-1-naphthol, to give a blue color, during a room temperature incubation that did not exceed 30 minutes. The 14 wells with black rings were antibody positive. Note the gradation of intensities ranging from very light to very dark dots. Work with cells from wells D7, D8, and D9, led to production of a monoclonal antibody that recognized a 24 Kdal polypeptide band in Dermacentor andersoni ova extract.



The vial containing the other half of the cells from wells D7, D8, and D9 of the original fusion plate was thawed, and its contents were diluted in complete growth medium containing fresh mouse thymocyte feeder cells at a concentration of  $1.3 \times 10^6$  thymocytes/ml, and these cells were placed into 60 wells of a microtiter plate. Within three weeks two wells of the 60 produced clones that screened positive for antibody to ova extract. Both of these wells were immediately expanded into larger wells of a 24 well plate. One positive well, B4, was cloned, but, the cells had not been counted properly so too many cells were added and all 60 wells contained positive clones.

The spent medium from the expanded wells was used as first antibody in an immunoblot to characterize the antibodies being produced by their binding to polypeptide bands of SDS-PAGE fractionated D. andersoni ova extract. All of the wells recognized a single polypeptide band that had migrated 42 mm from the origin and thus had an approximate molecular weight of 24 Kdal. The cells from these wells were inoculated into four flasks for use in ascites production and several vials of cells were frozen for future use.

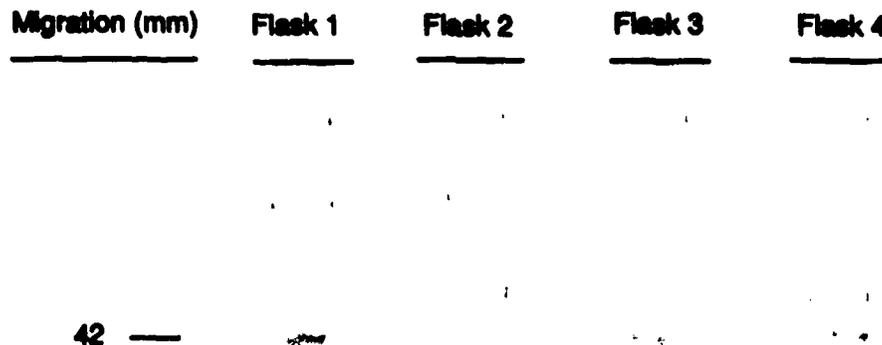
The cells in the flasks grew very well for two weeks, then the concentration apparently became too great, at which point the cells size decreased and they became picnotic. The cells were diluted with additional medium and looked healthier within 24 hours. To produce ascites the cells were concentrated, washed, and  $4 \times 10^5$  cells/ml were injected IP into BALB/c mice that had been primed with pristane two weeks earlier. Retrospectively, this was about one fifth of the cells recommended for injection. Four days prior to immunizing the mice IP.

an immunoblot was performed on medium from the flasks of cells used to immunize the mice and the antibody in the medium recognized a single band that had an approximate molecular weight of 24 Kdal. A photograph of these immunoblot strips can be seen in figure 27. A total volume of 60 ml of ascites was collected prior to euthanizing the five mice. The ascites had a Dot-ELISA titer of 1:8,192 to D. andersoni ova extract. Immunoblot characterization of the ascites showed approximately seven very weak diffuse bands instead of the expected single strong band at 42 mm which had an approximate molecular weight of 24 Kdal.

The fusion performed using spleen cells from mice immunized with D. andersoni SGA, immunization regimen III, yielded four wells out of 120 plated, that produced antibodies which recognized epitopes on either or both D. andersoni SGA or D. andersoni ova extract. Plate "A" had only well D4 (AD4) positive. This well was immediately expanded into a large volume. The antibody from this clone reacted only with SGA dots and recognized at least four bands in an immunoblot against D. andersoni SGA: one very dark narrow band at the very top of the gel and three faint bands that ran together just below the top band, which had molecular weights between 200 and 300 Kdal. Cells from the expansion wells were frozen.

Plate "B", of this fusion, had three wells, G7, G8, and E11, which produced antibodies that reacted more strongly with SGA dots, but they also reacted with ova extract dotted disks. All four wells were expanded and formal cloned. Well G7 failed to produce clones in two formal clone attempts. Well E11 produced clones in 108 of 120 wells, but, only one well out of 26 single clone wells was weakly positive

**Figure 27. Immunoblot characterization of monoclonal antibodies to Dermacentor andersoni ova extract (Regimen I).**



**Note:**

Approximately seven micrograms of the antigen, Dermacentor andersoni ova extract, was loaded per lane on an SDS-PAGE gel. This was electrophoresed under reducing conditions at 30 milliamperes until the running front reached 11.5 cm. It was then electroeluted to nitrocellulose a. 150 milliamperes for 18 hours.

First antibody was 100 microliters of medium from each flask of cells.

Second antibody was goat anti-mouse IgG-HRPO diluted 1:200.

Maximum reaction time with substrate was 30 minutes.

when screened with SGA. The first formal clone of well G8 failed, but in the second attempt 91 wells out of 110 had clones. Twenty four of these wells appeared to contain single clones. Twenty one of the single clone wells screened positive by Dot-ELISA for antibody to SGA. The first formal clone of well AD4 was successful with clones in 50 wells out of 120 plated. Seven wells, which appeared to contain single clones, were screened for antibody. Two wells, plate "A" well D10 and plate "B" well F7, were strongly positive. At the same time, the four originally positive wells which had each been expanded, screened positive. The cells from these expanded wells were harvested and frozen in aliquotes containing  $4-5 \times 10^6$  cells/ml.

Well F7 from the formal clone of original well AD4 was formal cloned resulting in 76 out of 120 wells containing clones. Dot-ELISA screen showed 67 of the 76 were positive for antibody to SGA. Two of these wells, G2 and F2, from the second plate were expanded. Wells G7 from the first cloning of AD4, and wells C10 and E4, from the second formal clone of original well BG8, were all expanded. The remaining positive wells from each plate were pooled and frozen.

The antibody from the expanded wells were characterized by immunoblot. Wells G7, G2, and F2, all descendants of original well AD4 had the same multiband pattern of approximately nine polypeptide bands covering a molecular weight range of 200 Kdal to 300 Kdal, with the five major bands having molecular weights of:  $\geq 300$ , 257.5, 250, 234, and 222.5 (illustrated in figure 28). Wells C10 and E4, descended from the original well BG8, had the same multiband pattern of approximately 17 polypeptide bands with ten major bands having molecular weights of:

≥ 300, 257, 234, 218, 200, 116, 100, 66, 64, and 47 Kdal which can be seen in figure 28.

#### Monoclonal Antibody Purification

The medium, from all the wells producing antibodies to D. andersoni SGA and with the same ancestral well in the original fusion plate, were pooled to create two pools, called AD4 and BG8. The cells from these wells were placed into corresponding pools which were cultured in two flasks each, for a period of about seven weeks. Spent medium was collected from the flasks each time new medium was added until each pool contained approximately 450 ml of spent medium. The spent medium which contained the desired antibodies was frozen at -20C. By seven weeks the cells for pool BG8 stopped producing detectable antibody to D. andersoni SGA. Pool BG8 was concentrated by ultrafiltration to a volume of seven milliliters with a protein concentration of 210 mg/ml.

Pool BG8 was processed, to purify IgG, by adapting the method of Bruck (Bruck et al., 1986), which was specifically designed for purification of monoclonal antibodies from ascites, using a DEAE Affi-gel Blue column. A 17 ml column of DEAE Affi-gel Blue was poured. The column was prewashed as suggested by Bruck et al. (1986) using 2 M guanidine HCl and 0.5 M NaCl, then reconditioned with 13 bed volumes of column buffer. Following dialysis against column buffer the seven milliliters of original sample, pool BG8, had a volume of nine milliliters. One third of this, with a protein content of 348.9 mg, was loaded on the column. Elution with 12 bed volumes of column buffer

**Figure 28. Immunoblot characterization of antibodies to Darmocentor andersoni salivary gland extract produced by hybridoma cells (Regimen III).**



**Note:**

Approximately ten micrograms of the antigen, Darmocentor andersoni salivary gland antigen (SGA), was loaded per lane on an SDS-PAGE gel. This was electrophoresed under reducing conditions at 30 milliamperes until the running front reached 11.5 cm. It was then electroeluted to nitrocellulose at 150 milliamperes for 18 hours.

First antibody was 100 microliters of medium from each expanded well.

Second antibody was goat anti-mouse IgG-HRPO diluted 1:400.

Maximum reaction time with substrate was 30 minutes. Antibodies from wells G7, G2, and F2, cloned from AD4, recognized the same nine bands; while well C10 and E4, clone from BG8 recognized the same 17 bands.

resulted in eleven, 17 ml aliquotes, which were pink, probably RPMI pH indicator, phenol red. This was followed by one aliquote that was colorless. The column was then eluted with 17 bed volumes of stepwise elution buffer which should selectively remove IgG. All 17 of the 17 ml aliquotes were a blue color. The absorbance of each aliquote was read on a UV spectrophotometer at 280 nm. The second aliquote, eluted with stepwise elution buffer, had the highest optical density (O.D.) reading, 0.8. This aliquote was concentrated by ultrafiltration to approximately one milliliter and stored at -20 C.

Following column regeneration, the second third of the sample was applied to the column. The column was eluted with column buffer, approximately 180 ml, until the eluate was no longer pink. The IgG was eluted with four bed volumes of stepwise elution buffer with each bed volume collected as a separate aliquote. Again the second aliquote had the highest O.D. reading, 0.65, at 280 nm. All four aliquotes were pooled and concentrated to a volume of 1.5 ml and stored at -20 C.

The final third of the pool, minus 0.2 ml saved for checking the beginning titer, was loaded on the column and processed the same as the second third. Again the second aliquote had the highest O.D. reading, 0.875. All elutions with stepwise buffer were a blue color, possibly due to unbound Cibricon Blue dye. One would expect to have removed all the originally unbound dye with the washes performed according to Bruck et al. (1986) and the samples processed by this point. Perhaps the RPMI caused some breakdown of the column. The three samples containing IgG were pooled and concentrated to a final volume of approximately three milliliters which had a protein concentration of 16.6 mg/ml.

This equates to 49.8 mg of IgG out of a total protein concentration of 1470 mg applied to the column for a 3.4% yield.

The solution, eluted with stepwise elution buffer, was dotted to nitrocellulose disks as antigen and tested by a direct Dot-ELISA technique which confirmed the presence of both mouse and bovine IgG. The BG8 pool, after dialysis, but, prior to application to the column, had a Dot-ELISA titer of 1:256 to D. andersoni SGA; but, only 1:4 after passage through the column and the final concentration step. Thus purification by this method resulted in a significant loss of reactivity and total IgG.

The ascites, produced using cells which had secreted a monoclonal antibody which recognized a 24 Kdal polypeptide band in D. andersoni ova extract, had a titer of 1:8,192 to D. andersoni ova extract, but reacted very weakly with approximately seven diffuse polypeptide bands when immunoblotted. Following the protocol of Bruck et al. (1986) for purification of monoclonal antibodies, three milliliters of ascites, with a total protein content of 83.4 mg, was pretreated and then applied to a DEAE Affi-gel Blue column, which had been prewashed with acetate-NaCl-isopropanol solution (Appendix II). Elution with three bed volumes of stepwise elution buffer, to remove IgG, gave a colorless solution. Two milliliter aliquotes were collected and checked spectrophotometrically at 280 nm for protein content. All of the eluate, including the void volume, was collected. The IgG came off in a rather narrow peak made up of approximately seven, two milliliter aliquotes out of 24 collected. Ten aliquotes or 20 ml were concentrated to approximately one milliliter and had a protein content of 10 mg for a

12% yield. Dot-ELISA technique using one microliter of this product as antigen and goat anti-mouse IgG-HRP0 at 1:100 as the antibody confirmed it was mouse IgG. The mouse IgG reacted with D. andersoni ova extract, but was not titered and the purity was not checked by SDS-PAGE.

#### Cross Reactivity

Adult ticks were routinely loaded on either rabbits, or guinea pigs, for propagation. In an attempt to decrease the costs involved in purchasing animals for this purpose, guinea pigs which had been used in immunization protocol II, and had been challenged with 100 A. americanum larvae, were reharnessed and loaded with a different species and stage, D. andersoni adults. The larval A. americanum which had been loaded on these guinea pigs as a challenge, after immunization with 50 micrograms of Sephadex G-200 peak 3 per 400 g body weight, had engorged and molted the same as larvae on naive controls. The adult D. andersoni engorged, but, 53% of the females turned a mahogany brown color and died.

To evaluate if this was due to the peak 3 immunization and stage specific activity or cross resistance between A. americanum and D. andersoni, two guinea pigs which had been used to feed large numbers of A. americanum larvae, two used to feed A. americanum nymphs, and one tick naive guinea pig were loaded with eight pair of adult D. andersoni per animal. None of these animals had been immunized. Twenty of the 32 females loaded on the pairs of previously infested guinea pigs died. Eleven of these females had fully engorged. The dead ticks all turned

a mahogany brown color. Only four females laid eggs. Of the eight females loaded on the control guinea pig, two died but were not engorged and four females laid eggs.

Serologic evidence of cross reactivity between D. andersoni and A. americanum was observed in immunoblot studies. One rabbit was loaded with 73 adult D. andersoni and a second rabbit was loaded with 41 adult A. americanum to obtain engorged ticks for SGA production and propagation. Sera from these rabbits had a Dot-ELISA titer of 1:128 to ova extract of the same species.

Immunoblot analysis was performed using the sera from these rabbits, diluted 1:32, as first antibody; electroeluted D. andersoni SGA strips and homologous species ova extract strips as antigens; and the second antibody was goat anti-rabbit IgG-HRPO diluted 1:200. The serum from the rabbit loaded with D. andersoni recognized 13 polypeptide bands in D. andersoni SGA with molecular weights ranging from 300 Kdal to 75 Kdal and eight polypeptide bands in D. andersoni ova extract with molecular weights ranging from 300 Kdal to 100 Kdal. Four of the polypeptide bands recognized in D. andersoni ova extract had the same molecular weights as bands recognized in D. andersoni SGA:  $\geq 300$ , 180, 110, and 100 Kdal. The serum from the rabbit loaded with A. americanum recognized nine polypeptide bands in D. andersoni SGA with molecular weights ranging from 280 Kdal to 58 Kdal, and six polypeptide bands in A. americanum ova extract with molecular weights ranging from 270 Kdal to 61 Kdal. None of the bands recognized in the Amblyomma americanum ova extract had the same molecular weights as bands recognized in D. andersoni SGA. Interestingly, the serum from the rabbit infested with

A. americanum recognized more polypeptide bands in the D. andersoni SGA than it did in the homologous species ova extract. Five of the bands it recognized in the D. andersoni SGA were the same molecular weight bands that were recognized by serum from the other rabbit: 280, 257, 200, 116, and 88 Kdal. The molecular weights of all polypeptide bands recognized by these rabbit sera are listed in table 19.

**Table 19. Immunoblot characteristics of rabbit serum showing cross reactivity with Dermacentor andersoni and Amblyomma americanum antigens.**

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Rabbit infested with 73 adult <u>Dermacentor andersoni</u>	
<u>Antigen:</u>	<u>Approximate molecular weight of bands recognized, in Kdal</u>
<u>Dermacentor andersoni</u> ova extra	≥300, 270, 240, 217, 180, 135, 110, 100
<u>Dermacentor andersoni</u> SGA	≥300, 280, 257, 200, 180, 140, 130, 116, 110, 100, 88, 81, 75
Rabbit infested with 41 adult <u>Amblyomma americanum</u>	
<u>Antigen:</u>	<u>Approximate molecular weight of bands recognized, in Kdal</u>
<u>Dermacentor andersoni</u> SGA	280, 257, 234, 218, 200, 116, 88, 84, 58
<u>Amblyomma americanum</u> ova extract	270, 255, 235, 180, 66.2, 61

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**Note:**

Immobilized antigens were fractionated on 12% SDS-PAGE gels, run under reducing conditions at 30 milliamperes until the running front reached 11.5 cm, then electroeluted to nitrocellulose strips at 150 milliamperes for 18 hours.  
 First antibody was sera from the rabbit listed diluted 1:32.  
 Second antibody was rabbit anti-guinea pig IgG-HRPO 1:200.  
 Maximum reaction time with substrate was 30 minutes.

## DISCUSSION

Laboratory studies of the phenomenon of resistance to tick infestation began with studies first reported by Trager (1939). Since that time, numerous investigators, utilizing various ixodid tick and host models, have attempted to elucidate mechanisms involved in acquired resistance to tick infestation and to artificially induce resistance in susceptible hosts (Willadsen, 1980; Wikel, 1982; 1983; 1984). Much of the early work involved studying acquired resistance and confirming its immunologic nature. Then research focused on inducing resistance by vaccination with crude whole tick extracts. This resulted in reports of success in inducing limited levels of resistance to tick infestation.

By the mid 1970s the need to refine this approach became clear. In order to develop a rationale for immunologic control of tick infestation, the mechanisms involved in the phenomenon of resistance must be understood. This requires the use of defined antigenic molecules (Wikel and Whelen, 1986). Beginning with the work of investigators such as Willadsen and Williams (1976) and the isolation and partial chemical characterization of one antigenic protein from B. microplus larvae, the direction of future research was set. Schleger and Lincoln (1976) used histochemical techniques and identified specific tick enzymes around the mouth parts of attached B. microplus larvae feeding on cattle. Zorzopulos et al. (1978) isolated and characterized phosphomonoesterases from B. microplus larvae and related them to possible sources in the ticks. Thus, the investigators listed,

although only a very small sampling, illustrate how some tick antigens have been isolated, purified, and chemically characterized; have shown specific enzymes were introduced into hosts by feeding ticks; and established the possible tick tissue source of specific antigens. More pieces of the puzzle of tick resistance have been identified.

Recent contributions advancing this effort were made by Whelen et al. (1984;1986) and Wikel and Whelen (1986) through introduction of immunoblot techniques for identification of many immunogenic tick molecules which may be involved in development of resistance and the Dot-ELISA technique for assessment of host immune responses to tick antigens.

Salivary gland antigens (SGA), gut antigens, and ova extract antigens, were studied to detect potential candidate molecules for use in an anti-tick vaccine capable of inducing resistance to tick infestation. Antigens utilized in this research were characterized by SDS-PAGE and immunoblot techniques. The salivary gland antigen (SGA), prepared from partially engorged female D. andersoni, was characterized by SDS-PAGE. The resulting silver stained gel contained 31 major polypeptide bands with approximate molecular weights ranging from  $\leq 10$  Kdal to 300 Kdal. This is the same number of major polypeptide bands that McSwain et al. (1982) reported finding in a similarly prepared SGA from partially engorged female A. americanum. Polypeptide profiles were obtained for salivary glands of female A. americanum throughout the engorgement period using Coomassie blue stained SDS-PAGE gels. The molecular weight ranges of these bands at  $\leq 30$  to  $\geq 218$  Kdal are similar to those observed in D. andersoni SGA. Gill et al. (1986)

detected up to 45 polypeptide bands with molecular weights ranging from 14.4 to 130 Kdal in SGA prepared from female Hyalomma anatolicum anatolicum allowed to engorge for 96 hours. The increased numbers of bands, relative to that found in this research, could be due to the species differences, or due to electrophoresing on a 19 cm long, 7% separating gel instead of a 12 cm, 12% separating gel which would possibly allow better separation and therefore more accurate counting of individual bands. Shapiro et al. (1986) found polypeptide bands ranging in molecular weight from 14.5 to  $\geq 200$  Kdal in unfed adult Rhipicephalus appendiculatus SGA. Thus, both the number and molecular weight range observed for polypeptide components of D. andersoni SGA in this research are similar to that reported by other investigators for other ixodid SGA. The number of polypeptide bands reported are obviously minimum numbers of polypeptides as tissues are composed of hundreds of polypeptides, many with the same or similar molecular weights.

Gut antigens were shown to induce resistance to tick infestation (Allen and Humphreys, 1979; Ackerman et al., 1980; Johnston et al., 1986). For this reason, gut antigens were investigated in this research. Three different gut antigens were prepared and characterized. Amblyomma americanum whole gut extract, BBF, and 27,000 x g supernatant had polypeptide bands ranging in molecular weight from approximately  $\leq 10$  to 158 Kdal. Shapiro et al. (1986) prepared gut extract from Rhipicephalus appendiculatus, electrophoresed it under reducing conditions using SDS-PAGE and electroeluted it to nitrocellulose. Serum from a guinea pig, expressing acquired resistance, was

incubated with the nitrocellulose. The antibody, from the serum of the guinea pig expressing acquired resistance, binding to nitrocellulose was localized using  $^{125}\text{I}$  labelled protein A and detected by autoradiography. The bands observed had molecular weights of 16, 20, 31, and 35 Kdal. These molecular weights fall within the range obtained for polypeptides found in A. americanum gut antigens in this research. Polypeptides in gut antigen could be recognized by specific host antibody if they, or similar polypeptides, had been injected into the host by regurgitation or transfer through tick salivary glands. Alternatively, these could be the result of cross reactivity to antigens with similar epitopes.

Immunoblot analysis, using serum from immunized animals and 27,000 x g supernatant as antigen, yielded patterns in which from two to 16 individual polypeptide bands were recognized. All of the bands recognized had molecular weights greater than the 35 Kdal noted by Shapiro et al. (1986). This may be due to a host response difference or variation in electrophoresis technique.

A third tick antigen prepared and characterized was ova extract. Resistance to tick infestation can be induced by the administration of whole ova extract (Dr. Stephen K. Wikel, 1985 - personal communication). Use of primary tick-tissue culture cells, derived from A. americanum ova, induced a significant degree of resistance to infestation by both adult A. americanum and adult D. andersoni (Wikel, 1985).

Willadsen and Riding (1980) isolated and purified an antigen from both tick eggs and tick larvae which was characterized by its activity as a proteolytic enzyme inhibitor, more specifically, a trypsin-chymo-

trypsin inhibitor. Willadsen and McKenna (1983a) reported that the egg enzymes were not the same enzyme found in the larval stage, but, had similar activity and were immunologically cross reactive. Immunologic cross reactivity would justify the use of eggs which are a more efficient and easier to work with source of tick antigens.

The buffalo black stained SDS-PAGE gel of D. andersoni whole ova extract, electrophoresed under reducing conditions, contained 19 major polypeptide bands which had approximate molecular weights ranging from 36 to 292 Kdal. Wikel and Whelen (1986) utilized a similar extract, A. americanum ova extract. The A. americanum ova extract was used in immunoblot studies to detect polypeptide bands that would be recognized by sera from tick resistant guinea pigs. Illustrations show molecular weights of the polypeptide bands ranged from approximately 50 to  $\geq$  200 Kdal which is consistent with the range observed for the D. andersoni ova extract bands. No other reports were found in the literature where ova extract was characterized.

Since resistance to tick infestation can be induced by administration of the very complex protein mixture composing whole ova extract, fractionation was undertaken to clarify which components might be significant in inducing resistance to tick infestation. Whole ova extracts were fractionated into defined molecular weight fractions by ultrafiltration and Sephadex G-200 gel filtration chromatography techniques. The resulting ultrafiltration fractions,  $\geq$  100 Kdal fraction and 30-100 Kdal fraction, contained polypeptide bands with molecular weights ranging from 39.5 to 278 Kdal and 35.5 to 92.5 Kdal, respectively. The Sephadex G-200 gel filtration chromatography

fractionation yielded three peaks: peak 1 contained polypeptide bands with molecular weights ranging from 70 to 260 Kdal with a trailing band at 46 Kdal, peak 2 bands had a range of 53 to 67 Kdal, and peak 3 bands ranged from 45 Kdal down.

Skin testing was performed with ixodid whole ova extracts and D. andersoni ova extract ultrafiltration  $\geq 100$  Kdal and 30-100 Kdal fractions on three guinea pigs. Distinctive differences were noted in the type and degree of reactions between the two previously infested guinea pigs. Guinea pig 2378, previously infested with larvae, had more immediate type hypersensitivity reactions than guinea pig 2379, previously infested with nymphs. Both animals had an immediate type hypersensitivity reaction to the 30-100 Kdal fraction but 2378 had a stronger reaction, 1.7 cm<sup>2</sup> compared to 2379 with an area of 0.9 cm<sup>2</sup>. This would be consistent with histologic findings by Riek (1962) that B. microplus larvae infestation resulted in a more severe reaction than nymphs on resistant cattle. The whole ova extract from D. andersoni did not apparently cause an immediate type hypersensitivity reaction but both D. albipictus and A. americanum whole ova extract did. One explanation might be the presence of circulating antibody which complexed with the antigen and prevented it from reacting with homocytotropic antibody which would lead to an immediate hypersensitivity reaction.

The beginning of intense delayed type hypersensitivity reactions were observed at six hours. At 24 hours unexpectedly strong, non-specific reactions observed on the naive control, especially those to D. andersoni 50-100 Kdal fraction and D. albipictus whole ova extract.

made interpretation of reactions on previously exposed guinea pigs difficult. This could be due to pharmacologically active components such as prostaglandins or histamine. Another possibility would be presence of a bacterial toxin. Delayed type hypersensitivity reactions appear to have occurred to every immunogen tested in the previously exposed animals except for D. andersoni  $\geq$  100 Kdal fraction, antigen 2, when reactions are compared to the same site at earlier readings. Dermacenter andersoni  $\geq$  100 Kdal fraction, antigen 4, induced the most severe reactions by size in all three animals with erythema, induration, and a white necrotic center observed in the previously exposed animals. Again, except for the reaction to D. andersoni whole ova extract, guinea pig 2378 had larger areas of reaction than guinea pig 2379. This may be a result of exposure to larger numbers of ticks, since nymphs are loaded in smaller numbers than larvae due to their size. Thus although the saliva exposure may be greater from nymphs individually, the exposure from larvae could be collectively more or qualitatively different than nymphs.

Riek (1962) reported skin testing cattle with antigens, from Boophilus microplus larvae, which gave immediate type hypersensitivity reactions only in resistant animals. There was no direct correlation between this reaction and degree of resistance. Prausnitz-Kustner reactions confirmed the involvement of homocytotropic antibody in the immediate type hypersensitivity reactions, but their size did not correlate with degree of resistance (Riek, 1962). Because the reactions of the naive control guinea pig were not observed at one hour, it is not possible to say the findings of this research agree with those

of Riek (1962).

The findings in this study agree with those of Wikel and Osburn (1982) in that both immediate and delayed reactions were observed when resistant cattle were skin tested with D. andersoni SGA. Amblyomma salivary gland antigens induced both immediate and delayed type hypersensitivity reactions in cattle previously infested with A. americanum (George et al., 1985). Perhaps this pattern is characteristic for some tick antigens, but Willadsen et al. (1978) isolated two allergens from B. microplus larvae that induced only immediate hypersensitivity reactions in resistant cattle. The size of these reactions correlated with tick yield. The larger the reaction, the fewer the ticks that were able to successfully engorge. The work of Willadsen et al. (1978) did not rule out other immune mechanisms such as antibody or cell mediated immunity functioning in resistance to tick infestation.

Histologic examination of skin test sites was performed to determine if the reaction to any antigen was similar to reactions reported for acquired resistance. The histology of the skin test sites showed eosinophils to be the dominant cell type observed for every antigen tested on guinea pig 2379, previously infested with nymphs. Interestingly, guinea pig 2378, previously infested with larvae, responded with a lower number of eosinophils and more lymphocytes than guinea pig 2379. The number of lymphocytes appears to correlate, as would be expected, with the degree of delayed type hypersensitivity reaction which is characteristic of cell mediated reactions. Some of these lymphocytes were probably also involved in the antibody response. Because of the time delay between the immediate hypersensitivity

reaction and obtaining biopsy material, the number of eosinophils did not correlate with the degree of the immediate hypersensitivity reaction. These findings differ from those of Gill et al. (1986) in which eosinophils were the dominant cell at two hours but by 48 hours neutrophils and mononuclear cells dominated the skin test sites on resistant rabbits injected with salivary antigens from Hyalomma anatolicum anatolicum. This is probably a result of timing of sample collection, but it could also be due to species differences. Binta and Cunningham (1984a) skin tested cattle with a fraction of Rhipicephalus appendiculatus larval extract. These antigens induced immediate hypersensitivity reactions which correlated to degree of previous tick exposure. The histology at 30 minutes post administration showed predominantly neutrophils, which are the most rapidly migrating granulocytes, with a few eosinophils, but by six hours the concentration of eosinophils had increased. Again, the results of this study differ because of the presence of neutrophils.

After skin testing showed the ova ultrafiltration fractions induced immunogenic responses, immunization studies were performed to ascertain if they would induce resistance to tick infestation. Resistance to tick infestation has historically been evaluated by its effects on infesting ticks. The effects vary widely and may be dependent on the host and tick species involved (Willadsen, 1980) as well as the degree of resistance. The effects range from rejection of ticks, with little or no damage, to death of the attached tick. Parameters used to evaluate resistance are: reduced numbers of ticks engorging, reduced engorgement weights, reduced quantities and viabil-

ity of eggs, prolonged feeding times, unsuccessful molting, and death of the tick (Willadsen, 1980; Wikel, 1983; 1984).

Immunization protocol I involved immunizing with ultrafiltration fractions of defined molecular weight ranges of A. americanum whole ova extract and challenging with 100 A. americanum larvae. Larvae from guinea pigs immunized with whole ova extract,  $\geq 100$  Kdal, and 30-100 Kdal fraction had reduced average engorged weights (0.05 level) compared to one exposure controls. Only whole ova extract and 30-100 Kdal fraction groups had significantly reduced numbers of larvae engorge (0.05 level). A significant parameter, that of viability, as determined by ability of engorged larvae to molt to nymphs, indicated resistance was induced by the 30-100 Kdal fraction. The 30-100 Kdal fraction immunized animals with 16% of larvae molting to nymphs had significantly fewer (0.05 level) than the one exposure controls with 55% molting, but more than the two exposure controls which had 9% molt. Thus, it is clear that immunization with the 30-100 Kdal fraction induced a degree of resistance comparable to the acquired resistance of two exposure controls, when this parameter was assessed.

Immunization protocol II guinea pigs were administered Sephadex G-200 peaks of A. americanum whole ova extract and challenged with 100 A. americanum larvae. This immunization protocol did not decrease the number of larvae that engorged on sensitized hosts. The treatment group immunized with Sephadex G-200 peak 2 had significantly fewer larvae (0.01 level) molt than the one exposure controls. Peak 2 immunized pair of guinea pigs were more resistant than the two exposure controls, expressing acquired resistance.

Immunization protocols I and II both involved vaccinating pairs of guinea pigs with fractions of A. americanum whole ova extract and then challenging each animal with 100 A. americanum larvae. In immunization protocol I only the 30-100 Kdal fraction induced resistance characterized by a decreased percent of larvae molting to nymphs. Two polypeptide bands with approximate molecular weights of 58 and 53.5 Kdal were found in the whole ova extract, but were totally missing in the  $\geq 100$  Kdal fraction. Both of these bands were highly concentrated in the 30-100 Kdal fraction. These molecular weight bands take on even more significance when the results of immunization protocol II are considered. Only peak 2 induced resistance characterized by a decreased percent of larvae molting to nymphs. The molecular weight range of polypeptide bands in peak 2 range from 53 to 67 Kdal, with a very heavy overloaded band which would correlate to the two found in the 30-100 Kdal ultrafiltration fraction. With the even narrower molecular weight range, these bands would be administered with fewer competing immunologic components. This could explain why peak 2 immunized guinea pigs were more resistant than two exposure controls.

Immunization protocol III animals were immunized with the same Sephadex G-200 peaks that were used in immunization protocol II at a rate of 20 micrograms per 250 grams body weight. In addition 20 micrograms of whole gut extract per 400 grams body weight was incorporated into this protocol. Another significant difference from protocol II was that the tick challenge was adult A. americanum rather than larvae. The results were that no significant differences were noted in any of the parameters when immunization treatment groups were

compared to one exposure controls.

Sephadex G-200 peak 2 plus gut did not induce an increased level of resistance in this protocol compared to one exposure controls. There are at least two possible explanations for this lack of effect. The easiest, although not substantiated, possibility is that this fraction induced a stage specific resistance. This may be supported by the fact that engorgement was unaffected and the impact was on molting in protocol II. Evidence of stage specific antigens were reported in several articles. Willadsen and Riding (1980) found a protease inhibitor in B. microplus larvae was missing in nymphs and adults. Further study of this enzyme (Willadsen and McKenna, 1983) showed that, contrary to first belief, it was different than the one found in eggs. Shapiro et al. (1986) reported that many adult tick salivary antigens were not found in larval and nymphal ticks. The other possibility is that addition of the heterogenous whole gut extract resulted in competition of so many immunogenic components that the immune response to the significant components was too weak to induce resistance.

No immunization studies were reported in the literature that utilized ova extract. The antigen which seemed like it should be most similar to ova was the antigen prepared by using tick-tissue culture cells. Wikel (1985) prepared primary tick-tissue culture cells from A. americanum ova. These cells were injected SC without adjuvant in guinea pigs that developed significant resistance to infestation with adult A. americanum and cross resistance to D. andersoni. This resistance was characterized by decreased engorgement weights, decreased ova production, and decreased viability. Although the antigen source

appeared similar to ova extract, the results are significantly different. Ova extract immunizations induced stage specific resistance to larvae and not to adults. Actually, the resistance pattern is very similar to that obtained by immunizing with 27,000 x g supernatant in protocol IV. This is logical as developing larvae probably are proportionally more gut tissue than any other organ.

Immunization protocol IV utilized A. americanum gut antigens. This specifically involved immunizing pairs of guinea pigs with either one microgram of brush border fragments (BBF) or ten micrograms of 27,000 x g supernatant. The responses/effects obtained in these guinea pigs were compared to those observed for the control guinea pig, immunized with PBS. This protocol returned to the concept that limiting the mixture of polypeptides incorporated into the immunization would result in stronger, more specific, responses that may be more effective in inducing resistance. An additional concept that precipitated this protocol involved the desire to remove the negative effects of resistance, skin lesions due to hypersensitivity reactions in the host, and replace them with reactions, such that the immunopathology would occur totally in the ectoparasite. This rationale requires the use of antigens not involved in acquired hypersensitivities reactions. This concept had been suggested by Ackerman et al. (1980).

The treatment group immunized with one microgram of BBF per 250 g body weight was not significantly different than the PBS control group in any of the parameters measured. The statistical analysis used compares mean values of the treatment group to the control group. This may mask what could be significant differences in this case as guinea

pig 2458 had all four adult female A. americanum fully engorge such that the average tick weight (0.34 g) was greater than that of the ticks from the PBS immunized control (0.24 g). All four of these ticks layed eggs and had an average egg mass weight of 0.16 g which was also greater than that of the four control ticks (0.09 g). Thus averaging the values obtained from guinea pig 2458, which were greater than control values, with the average tick weight of 0.07 g and average egg mass weight of 0.02 g, for the two ticks that layed eggs, from guinea pig 2457 masks potentially significant findings. This variation, between treatment group pairs, may be the result of variability of host immune response. Johnston et al. (1986) found that three of fifteen immunized cattle developed poor resistance while the other 12 developed intermediate to good resistance using the same immunization and immunization regimen. The low dose of BBF antigen used to immunize guinea pigs, dose response, may also have some bearing on these results.

The treatment group immunized with ten micrograms of 27,000 x g supernatant per 250 g body weight developed resistance characterized by significantly decreased tick engorged weight (0.05 level) and decreased egg mass weight (0.01 level) compared to the ticks from the PBS immunized control. What may be an even more important aspect of this resistance is that 50% of the adult female ticks and 37.5% of adult male ticks that engorged on the 27,000 x g supernatant immunized animals died, while none of the ticks on the other two treatment groups died. Three of the four female ticks and two of the male ticks died after engorging on the same guinea pig, 2459. One female tick died two

weeks after engorging and detaching. This may reflect the action of antibodies consumed with the blood meal as well as shared target antigens in male and female ticks. The dead ticks had turned a brown color and no ticks with a red color, due to gut rupture and the presence of host erythrocytes in the hemolymph as reported by Kemp et al. (1986), were noted. Death may have been due to gut damage less severe than that observed by Kemp et al. (1986) but histologic studies were not performed on the ticks.

Allen and Humphrey's (1979) utilized D. andersoni gut in preparation of two antigen extracts used to immunize guinea pigs. One antigen extract was prepared from midgut and reproductive organs while the second extract was prepared from all internal organs. Both were administered with Freund's complete adjuvant (FCA). Ticks which fed on guinea pigs immunized with the first extract had reduced engorged weights, reduced egg mass weights, and produced no larvae. Ticks loaded on guinea pigs immunized with extract II did not engorge nor lay eggs. Antigen I was also used to immunize calves. This resulted in a significant level of resistance to tick infestation (Allen and Humphreys, 1979).

The results of protocol IV, like those of Allen and Humphreys (1979), were reduced engorgement weights and reduced egg mass weights. The same degree of resistance was not obtained as eggs were produced and hatched to larvae. This is probably due to the lower concentration of antigens used in the immunizations and may also reflect not having used FCA. Allen and Humphreys (1979) reported the organs utilized in the extracts were obtained from ticks which had engorged for five days

because organs from unfed ticks had previously been found to be ineffective. Unfed ticks were used to prepare BBF and 27,000 x g supernatant. Perhaps the fractionated antigen compensated for lower enzyme and/or target antigen concentrations possibly found in unfed compared to fed tick gut. There is the possibility that use of midguts from partially engorged ticks would increase the effectiveness of these preparations as desired target antigens may have increased as a consequence of engorgement. Although it would seem that antibodies produced against host antigens contained in the tick gut would be undesirable.

Ackerman et al. (1980) prepared whole tick extract from unfed D. variabilis. Midgut soluble extract and midgut pellet were prepared from ticks which had engorged for five days. Rats were the hosts used in the study. Ticks that engorged on rats immunized with midgut soluble and midgut pellet had significantly reduced average engorged weight, egg mass weight, and reduced hatchability of eggs compared to the unimmunized and whole tick extract immunized controls. No tick fatality resulted from these treatments. The reduced engorged weights, reduced egg mass weights, and decreased hatchability agree with the findings of protocol IV. A major difference noted is that 50% of engorged females and 37.5% of males from guinea pigs immunized with 27,000 x g supernatant died. Further study with larger numbers of animals and ticks would be required to determine if this difference is due to the immunization or is due to differences in species of host and/or tick.

An interesting side point is that Bagnall (1978) reported rats did not develop acquired resistance to Ixodes holocyclus and were used for

feeding all stages of the tick for this reason. Being able to induce immunologic resistance in a species incapable of developing acquired resistance presents a very strong case for the use of gut antigens in a vaccine for immunologic control of tick infestation. This case is strengthened further by the possibility that immunopathology may be occurring in the tick rather than the host (Ackerman et al., 1980).

Ackerman et al. (1980) found that both particulate and soluble components prepared from midgut induced resistance. This agrees with immunization results using 27,000 x g supernatant (soluble) and BBF (particulate) from protocol IV. This is further supported by the results of Johnston et al. (1986) in which both extract and pellet individually and in combination induced resistance to infestation by B. microplus.

Johnston et al. (1986) prepared a whole tick extract of partially engorged, adult, female, B. microplus which was filtered to remove cuticle. The resulting extract must have been very similar to the antigen extract II prepared from all internal organs of D. andersoni by Allen and Humphreys. Johnston et al. (1986) immunized using both preparations with FCA given IM in the neck and the rump as well as SC over the ribs without adjuvant. This approach may result in stimulation of both cell mediated and antibody mediated defenses.

The results reported by Johnston et al. (1986) suggest that the extract was enriched by significant gut antigens as decreased engorgement weights were accompanied by gut damage. In a related article, Kemp et al. (1986) reported that up to 60% of adult females displayed gut damage. This resistance was apparently stage specific as it did

not affect larvae and it functioned quite differently than acquired resistance. Acquired resistance resulted in tick rejection, particularly larvae as infestation was beginning, and was characterized by hypersensitivity reactions and serous exudate at tick attachment sites. The mechanism involved is apparently complement dependent and antibody mediated as demonstrated through in vitro feeding of ticks with serum from immunized cattle (Kemp et al., 1986). The effect of this resistance was destruction of digest cells in the tick gut lumen as observed in tick histology (Agbede and Kemp, 1986).

Antibodies which recognized gut epitopes were stimulated by immunization with gut extract in protocol III and in protocol IV by immunization with BBF and 27,000 x g supernatant. These antibodies were identified by immunoblot analysis of serum from immunized and challenged hosts. No antibodies which recognized gut epitopes were observed in serum from animals with acquired resistance. No apparent pattern was detected such that any particular molecular weight bands in the gut antigens could be associated with induction of resistance.

Tick attachment site histology was studied for clues of resistance mechanisms that might be induced by the immunizations and by active infestation. Two types of white blood cells, eosinophils and lymphocytes, were regularly identified in the seven micrometer, paraffin embedded, sections from tick attachment site biopsies. Basophils were rarely noted in sections from protocols I or II. Epidermal thickening and eosinophil degranulation appeared to correlate with increased resistance except for the case of guinea pigs immunized with Sephadex G-200 peak 3, that showed no increase in resistance but had eosinophil

infiltration of the epidermis that resulted in considerable epidermal thickening. These findings agree with those of Rubaire-Akiiki and Mutinga (1980) that rabbits infested with Rhipicephalus appendiculatus had epidermal thickening and infiltration which were more marked in subsequent infestations. The infiltration, at tick attachment sites in subsequent infestations, was characterized by increased numbers of eosinophils, accompanied by degranulation, and increased numbers of lymphocytes. Vesicle formation was also noted for larvae and nymphs in later infestations (Rubaire-Akiiki and Mutinga, 1980). Riek (1962) reported that the histology of larval Boophilus microplus attachment sites on resistant cattle were characterized by intense infiltration of polymorphonuclear leukocytes, which were predominantly eosinophils. This cellular response was accompanied by pustule formation and serous exudate. Schleger et al. (1976) studying the histology of Boophilus microplus attachment sites on cattle reported dermal infiltration by eosinophils and formation of vesicles in resistant animals. Neutrophils were noted in higher numbers but were not quantitated. Basophils were observed in 0.5 micron sections, but their numbers were insufficient to warrant quantitation (Schleger et al., 1976). Matsuda et al. (1985) found mice responded to repeated infestation with Haemaphysalis longicornis with significantly increased numbers of mast cells and eosinophils, but could not find significant numbers of basophils. Allen (1973) found eosinophils to be a notable feature in resistant guinea pigs infested with D. andersoni larvae. Allen (1973) was the first to report the presence of basophils at tick attachment sites on resistant guinea pigs with eosinophils and basophils composing

nearly equal portions of the dermal infiltrate.

Few neutrophils were identified, with means ranging from zero to 3.2, at tick attachment sites, in either protocol I or II. In contrast Gill and Walker (1985) found neutrophils to be the major component of the cellular infiltrate at H. a. anatolicum attachment sites on both susceptible and resistant rabbits from 24 to 144 hours post attachment. Tatchell and Moorhouse (1970) also reported high concentrations of neutrophils in Rhipicephalus sanguineus attachment sites on dogs and further indicated seeing this type of response with a wide range of species of ticks. Brown and Knapp (1980a) found neutrophils to be the dominant cell type and eosinophils the second most prevalent cell type within feeding lesions of A. americanum larvae on susceptible guinea pigs at 24 hours and all cell types decreased in number after 48 hours.

The presence of large numbers of neutrophils and their degranulation in the cellular response to tick infestation has been directly related to lysis of host cells and collagen destruction resulting in development of the the tick feeding lesion (Tatchell and Moorhouse, 1970; Berenberg et al., 1972, Gill and Walker, 1985). It is possible that in some tick-host associations an inflammatory response occurs and neutrophils are the initial granulocytes to arrive. Other cell types move into the area in waves in response to chemotactic factors thus replacing neutrophils as the dominant cell type. This theory or the simple degranulation of neutrophils could explain why so few neutrophils were observed in this research and eosinophils were the primary cell type identified.

Brown and Knapp (1981) reported significantly different histologic

findings for A. americanum larvae infesting hypersensitized guinea pigs. At 12 hours post attachment, basophils were the primary cell type observed in feeding lesions, but by 72 hours eosinophils were the dominant cell type. No neutrophils or erythrocytes were noted in the feeding lesions.

It is unfortunate that the Brown and Knapp (1981) study did not include histology of sections from nonresistant hosts infested in parallel with the resistant guinea pigs for response comparison. The cellular response observed in the two exposure controls of protocol I compared favorably with those observed in protocol II, but the one exposure controls were quite different in the numbers of eosinophils and lymphocytes noted. The larval ticks were allowed to engorge for five days in both protocols, but the mean engorgement weights of one exposure controls from protocol I of  $10.4 \times 10^{-4}$  g is considerably heavier than the mean weight of protocol II, one exposure controls at  $7.7 \times 10^{-4}$  g. Trager (1939) noted a great variability in the infesting power of different batches of ticks and of the members of the same batch at different times. Thus the variability observed, between protocol I and II one exposure controls, may reflect a difference in willingness to engorge which could affect the quantity of tick antigens to which the host was exposed.

Allen (1973) was the first to identify and report the presence of basophils and cutaneous basophil hypersensitivity (CBH) type of cellular response in guinea pigs resistant to infestation by D. andersoni. Since that time numerous investigators have reported the presence of basophils and CBH reactions in a wide range of ixodid-host

combinations (Willadsen, 1980; Wikel, 1982; 1983; Wikel and Whelen, 1986). Basophils were not identified in significant numbers in any tissue sections in this study. Allen (1973) noted basophils were demonstrable in thin sections of Epon-embedded skin, but were not revealed by more traditional histological techniques. Both Bagnall (1978) and Brown and Knapp (1981), in contrast to Allen (1973), reported identifying basophils in eight micrometer thick paraffin sections using modified geimsa stains. Although it is possible processing technique may have influenced the identification of basophils, this seems unlikely. Biopsies were placed into cold Karnovsky's fixative as soon as they were collected. This should have adequately fixed any basophils present. Staining with the nonaqueous geimsa according to Wolboch's modification should also have differentially stained and stabilized white blood cells including basophils. The few basophils noted were well stained and classic in appearance, with dark blue granules about the same size as eosinophil granules, usually too dense to see the lobed nucleus clearly. A more likely explanation for not identifying basophils is the timing of specimen collection. Since eosinophils are feedback regulators of basophils and mast cells, the presence of eosinophils may suggest the earlier presence of basophils. The cellular response is a dynamic or continually changing phenomenon. As suggested by Allen et al. (1977) basophils infiltrate due to specific stimuli, degranulate, thus producing a stimulus which results in infiltration of a new wave of eosinophils. This would agree with the finding of Brown and Knapp (1981) that in resistant guinea pigs basophils were the primary cell type at 12 hours post attachment but

were replaced as the dominant cell by eosinophils at 72 hours post attachment. Thus biopsies, in this research, taken 120 hours post attachment would reasonably have eosinophils as the dominant cell type.

The cross section of the attached A. americanum larva shown in figure 8 allows a clear view of the gut contents. Host cellular components are seen including eosinophil granules. This conflicts slightly with the description of Brown and Knapp (1980) in that although the contents are becoming amorphous, no red mass indicating rapid engorgement of erythrocytes was noted.

Comparing the histology of guinea pigs immunized in protocol I to those from protocol II, one notes a significant difference in the number of lymphocytes and other basophilic cells observed. This may be the result of differences in the constituents of ultrafiltration fractions compared to Sephadex G-200 peaks. Potentially, some tick proteins, such as the enzymes described by Willadsen and McKenna (1983b), bind nonspecifically to other proteins and hydrophobic supports. These may have been lost on the Sephadex G-200 column but would have been recovered by ultrafiltration, as the filters surface was forcibly rinsed using a pipet. Again there is the possibility that the quantity of tick antigen injected during the challenge may have varied between the two protocols and the difference is a dose dependent response. With the many variables in the complex, multifaceted host-parasite relationships, variation in techniques used by investigators, and the dynamic nature of immune response mechanisms, interpretation and correlation of histologic findings is very difficult.

The role antibody might play in resistance was investigated by

determining titers and comparing them to histologic cellular responses and expressed resistance. Serum from animals immunized in protocol I had Dot-ELISA titers to A. americanum ova extract that ranged from 1:4,096 to 1:65,536. Guinea pig serum from the animals immunized in protocol III had titers to A. americanum ova that ranged from 1:512 to 1:8,192 and to A. americanum gut extract that ranged from 1:128 to 1:4,096. These titers indicate the extracts used for immunization were capable of stimulating antibody production. However, antibody did not correlate to tick resistance. Guinea pigs immunized with 30-100 Kdal fraction expressed the greatest degree of resistance in protocol I, but had only mid range titers of 1:16,384. Guinea pigs immunized with whole ova extract had titers of 1:32,768 and 1:65,536, but displayed less resistance than 30-100 Kdal immunized guinea pigs. None of the immunized guinea pigs in protocol III expressed resistance despite having high titers to both ova and gut antigens. The lack of a direct relationship between circulating antibody and resistance agrees with the findings of Whelen et al. (1986). Although resistance as expressed by engorgement weights did not change significantly between the second and fourth infestations, mean titers continued to increase through the fourth infestation to a peak of 1:1,400. Then the titers rapidly dropped to 1:300 where they stabilized. Willadsen et al. (1978) also reported that the correlation between tick yield and hemagglutination titers was much weaker than that observed with two allergens and the size of the immediate hypersensitivity reaction they elicited in previously exposed cattle. Although circulating antibody has been shown to contribute to resistance to tick infestation by passive

transfer studies (Bagnall, 1975; Roberts and Kerr, 1976; Brossard and Girardin, 1979; Brown and Askenase, 1981) and by selective depletion of B-lymphocytes (Wikel and Allen, 1977) it has been established by other workers that other mechanisms are involved in expression of resistance (Willadsen, 1980; Wikel, 1982; 1983; 1984).

Homocytotropic antibodies have been shown to be related to resistance in cattle (Riek, 1956; Binta et al., 1984) and in rabbits (Boese, 1974; Brossard and Girardin, 1979; Brossard et al., 1982; McGowan, 1985). Homocytotropic antibodies bound to host tissues would not be detected by serum antibody titers. A higher level of resistance was attained by transfer of lymph node cells (Bagnall, 1975; Wikel and Allen, 1976) than by transfer of serum. This indicates a cell mediated immune mechanism is involved in resistance to tick infestation, perhaps to a greater degree than circulating antibody. Since neither animals receiving lymph node cells nor animals receiving serum from immune hosts express a level of resistance comparable to the donor that actively acquired resistance, it is apparent that either other mechanisms are involved (Wikel and Whelen, 1986) or that synergism results in a greater degree of resistance.

Immunoblot analysis was performed using serum from immunized animals in an attempt to identify immunogenic components of tick extracts that might be associated with resistance. Serum was collected, from animals which had been immunized in protocols I, II, III, and IV and then infested with A. americanum. The antibody specificity was analyzed by immunoblotting. The immobilized antigen utilized in immunoblot analysis was the whole extract used for immunization except

for protocol IV, where 27,000 x g supernatant was substituted for whole gut extract. The molecular weights of bands recognized by the serum of different animals was compared to establish possible patterns which could be associated with resistance. Variations in both the numbers and molecular weights of polypeptide bands recognized, by pairs of animals in the same treatment group, limited the possibilities. Comparison of these possibilities, between groups of resistant and susceptible animals, resulted in no patterns which could be specifically associated with resistance to tick infestation.

Because of the large number of animals, molecular weight bands recognized, antigens immobilized, and the combinations of each of these involved in a comparison only a very small example from protocol IV will be listed here for demonstration purposes. Guinea pigs 2457 and 2458 were both immunized with BBF, challenged with 100 A. americanum larvae, and then bled to obtain the serum used in immunoblot analysis. Serum from guinea pig 2457 recognized two bands with molecular weights of 148.0 and 60.4 Kdal, while serum from guinea pig 2458 recognized seven bands with molecular weights of 156.0, 148.0, 137.0, 133.0, 125.0, 113.0, and 60.8 Kdal. Control guinea pig, 2462, was immunized with PBS and recognized one band with a molecular weight of 60.8 Kdal. Whelen et al. (1984) and Wikel and Whelen (1986) reported sera collected from guinea pigs prior to infestation recognized a 65 Kdal polypeptide in D. andersoni larval extract and in A. americanum ova extract. This may be similar to the 60.8 Kdal band observed in this research. The 60 Kdal band is assumed to be a nonspecific band because it is recognized by serum from a control animal or at least to not be

associated with resistance since this animal expressed no resistance. That leaves only the 148 Kdal band for guinea pig 2457, which expressed some degree of resistance, to be compared to its treatment mate, guinea pig 2458. Serum from both animals recognized the 148 Kdal protein, but guinea pig 2458 expressed no resistance, having had ticks engorge to a larger size and lay larger egg masses than the control guinea pigs. This logic pattern was applied to all blot analysis data and resulted in no conclusive or even suggestive patterns to associate polypeptide bands with resistance.

Only a few studies using immunoblotting to either detect or characterize immunogenic components of tick extracts have been published. Gill et al. (1986) used serum from resistant rabbits to detect immunogenic polypeptides in SGA from H. a. anatolicum that might be involved in resistance. This study demonstrated a large number of tick salivary components were immunogenic which agrees with the findings of this research. Three immunogenic heavy molecular weight bands were extracted, partially chemically characterized, and skin tested. All three elicited immediate hypersensitivity reactions indicating the possible role of homocytotropic antibodies, IgE or IgG1, and two elicited delayed type hypersensitivity reactions indicating cell mediated mechanisms. Although this research did not show a direct relationship between any of these immunogenic bands and resistance, the skin reactive fractions might play a role, "as yet undefined", in host resistance.

Shapiro et al. (1986) used the serum from one highly resistant guinea pig to identify immunogenic components in extracts of adult R.

appendiculatus salivary gland, gut, and attachment cement as well as larval and nymphal extracts. The results were that unique components were recognized in every extract, with some components with similar molecular weights being recognized in several extracts. Shapiro et al. (1986) reported that many different immunogenic tick molecules must be inoculated into infested hosts and suggested resistance is a complex phenomenon probably elicited by several tick antigens.

It is highly unlikely, given the diversity of host response obtained in immunized hosts from protocols I, II, III, and IV that tick antigens significant to resistance could be determined from use of a single resistant host animal. Large numbers of polypeptide bands recognized by immunoblot analysis of various tick extract preparations confirms numerous polypeptides are involved in resistance (Gill et al., 1986; Shapiro et al., 1986). Although their assumptions may be correct, actually the only thing proven is that there are many immunogenic components in these extracts which may be injected into the host during engorgement or may contain epitopes that are similar to those on injected antigens. Immunoblot analysis in this research also demonstrated numerous bands were recognized in tick extracts, but they could not be directly associated with resistance. Skin testing performed by Gill et al. (1986) with three salivary gland extract proteins may be a stronger indicator of significant immunologic responses than circulating antibodies which have been shown in this study and by others (Willadsen et al., 1978; Whelen et al., 1986; Johnston et al., 1986) not to correlate directly to degree of resistance to tick infestation.

Whelen et al. (1984) and Wikel and Whelen (1986) performed immunoblot analysis using serum from guinea pigs, rabbits, and cattle that had been infested with either D. andersoni or A. americanum and used them in immunoblot analysis to detect immunogenic components in D. andersoni larval extract and A. americanum ova extract. Post infestation sera from all host animals except a Brahman calf recognized additional epitopes not recognized prior to infestation. Two observations reported by Whelen et al. (1984) and Wikel and Whelen (1986) agree with findings in this research: (1) Different infested host animals of the same species had serum that recognized different components in the same tick extracts. (2) Animals infested with one species of tick recognized epitopes in various extracts of the homologous species, and also some epitopes in extracts of other ixodid species.

Cross reactivity was demonstrated by immunoblot analysis and sequential infestations. Immunoblot analysis with serum from two rabbits, one infested with adult D. andersoni and one infested with adult A. americanum, confirmed the findings of Whelen et al. (1984) that hosts infested with one of these species will produce antibodies that recognize epitopes in antigens from the other ixodid species. The polypeptides recognized by cross reactivity had different molecular weights than those recognized in the homologous system (Whelen et al., 1984). Serum from the D. andersoni infested rabbit was immunoblotted against homologous antigens, D. andersoni SGA and ova extract. Serum from the rabbit infested with A. americanum was immunoblotted with A. americanum ova extract and D. andersoni SGA. As might be expected,

because of the natural injection of salivary antigens by engorging ticks, rabbit serum, from the rabbit infested with D. andersoni, recognized more antigens, 13 bands, in D. andersoni SGA than in D. andersoni ova extract, eight bands. It was surprising to find that the serum from the A. americanum infested rabbit recognized more components in D. andersoni SGA, nine bands, than it recognized in the A. americanum ova extract, six bands. Unlike the results of Whelen et al. (1984) five out of nine of the components recognized by serum from the A. americanum infested rabbit in the D. andersoni SGA had the same molecular weights, 280, 257, 200, 116, and 88 Kdal, as bands recognized by serum from the D. andersoni infested rabbit. This cross reactivity may be indicating the presence of some highly conserved epitopes in these two ixodid genera which are considered distantly related. The identification and isolation of antigens with common epitopes in divergent ixodid genera, which may be involved in the development of resistance, could be a significant step in development of a functional vaccine with broad coverage.

Additional evidence of not only cross reactivity but more specifically cross resistance between D. andersoni and A. americanum was obtained through an infestation study. Guinea pigs which had previously been heavily infested once with either A. americanum larvae or nymphs were infested with eight pair of adult D. andersoni. Sixty-two percent of the adult, female, D. andersoni infesting guinea pigs previously infested by A. americanum died after engorging and only 12.5% laid eggs. One tick naive guinea pig was used as a control. Eight pair of adult D. andersoni were loaded at the same time as the

previously infested guinea pigs were re-infested. Two or 25% of the females from the control died, but had not engorged, and four or 50% of the females laid eggs. The effects of a relatively high level of resistance were apparent, but unfortunately the mechanisms involved were not apparent. It is possible that the mechanism involved could be humoral. Since IgG has been reported, by Ackerman et al. (1981), to cross tick gut membranes, a low concentration of antibody would allow the ticks to engorge, then cross the gut where it could act on multiple target systems. By acting on the reproductive system fertility could be affected in some ticks, but if multiple systems were attacked the result could be death in other ticks. These findings conflict with the findings of McTier et al. (1981) that A. americanum resistant guinea pigs were not cross resistant to D. andersoni.

Trager (1939a) was the first to report cross resistance, as demonstrated by decreased engorgement weights for D. variabilis and D. andersoni. George et al. (1985) also demonstrated cross reactivity by skin testing cattle, previously infested with low numbers of A. americanum, with SGA from A. americanum, A. cajenense, and D. andersoni. The SGA from Amblyomma species both elicited immediate and delayed reactions, while that from D. andersoni resulted in only immediate reactions. Yet, Wikel (1985) stimulated a significant degree of cross resistance to D. andersoni by immunizing with A. americanum ova derived primary tick-tissue culture cells. From this it appears that the extent of exposure relating to a dose dependent response may affect the degree of cross reactivity. In contrast to these reports, Wagland et al. (1985) reported that cattle infested with both Boophilus

microplus and Haemaphysalis longicornis expressed no cross resistance impacting the other tick population. Thus, although in this study, strong cross resistance was demonstrated, it appears that it is limited to certain tick genera combinations. One possibility is that antigens are expressed at different levels in different tick species. The ability to induce cross resistance by immunization with tick-tissue culture cells (Wikel, 1985) strongly supports the concept of conserved epitopes and the potential for developing broad coverage through immunization. The factors involved and limitations require further investigation. Cross reactivity will be better understood as the mechanisms involved are defined. Monoclonal antibodies may be useful tools for helping define these mechanisms by identifying highly conserved epitopes.

Monoclonal antibodies were produced to D. andersoni antigens. Spleen cells from mice that had been injected with D. andersoni ova extract were fused with NS-1 myeloma cells. This fusion resulted in 14 wells out of 60 containing hybridoma cells that produced antibodies to D. andersoni ova extract. Three wells were pooled, with half of the cells frozen and half of the cells were subcloned. All of the cloned cells were lost to fungal contamination. This points out not only the need for aseptic technique but the importance of freezing cells at each step. The frozen cells were thawed and placed into wells with feeder thymocytes. Two wells out of 60 contained hybridoma cells producing antibody to D. andersoni ova extract. This was essentially the same as performing a formal clone procedure. Both of these wells produced antibody which recognized a single polypeptide band with a

molecular weight of 24 Kdal when characterized by immunoblotting with D. andersoni ova extract. Cloning twice is recommended by Goding (1983). According to Collier and Collier (1983), with up to 5% of wells in a first subclone showing growth, up to 75% of wells in the second subcloning can show growth and still have a 99% probability of being monoclonal. Technically these cells should be cloned one more time to call the antibody truly monoclonal, although based on reactivity with a single polypeptide band they are functionally monoclonal. This is the first report of a laboratory producing monoclonal antibodies to proteins of tick origin. Specifically, these are the first monoclonal antibodies produced specific for D. andersoni antigens.

A fusion was performed with spleen cells from mice immunized with D. andersoni SGA and NS-1 cells. This fusion resulted in four wells out of 120 wells plated that contained hybridoma cells which produced antibody to D. andersoni SGA. Well AD4 was formal cloned and expanded. The first formal clone had 50 out of 120 wells containing clones, 42%. Well F7 from this clone was formal cloned with 76 out of 120 wells containing clones, 63%. According to Goding (1983) and the Poisson distribution, being 37% of the wells contained no clones there was a reasonable probability that antibody produced in any one well would be monoclonal. The table produced by Collier and Collier (1983) indicated that with 42% of the wells showing growth in the first subcloning only 45 to 53% of the wells in the second subcloning could show growth for a 95% probability of monoclonality. Interestingly immunoblot analysis showed that antibody from the original well recognized the same bands in SGA as two wells expanded from the second subcloning. This multi-

band pattern, shown in figure 28, must represent a monoclonal antibody which recognized one epitope common to all of these components.

A second well, G8 in plate B or BG8, was subcloned. The first subclone failed, with no wells showing sustained growth. The second subclone attempt resulted in 91 out of 110 wells showing growth, 83%. No further subcloning was performed, but two wells, C10 and E4, were expanded. Antibodies, from the expanded wells, were used in immunoblot analysis and the antibody from both wells recognized the same bands as can be seen in figure 28. This multiband pattern contains more bands than were seen in wells originating with AD4, but intuitively it seems unlikely that two wells could contain exactly the right cells to produce antibodies that would recognize this many bands in an identical pattern.

The antibody from the AD4 pool cells recognized five polypeptide bands in D. andersoni SGA with molecular weights of 300, 257, 250, 234, and 222 Kdal. The antibody from BG8 pool cells recognized polypeptide bands with molecular weights of 300, 257, 234, 218, 200, 116, 100, 66, 64, and 47 Kdal. Polyclonal serum from a rabbit infested with adult D. andersoni recognized five bands with the same molecular weights recognized by BG8 pool cells: 300, 257, 200, 116, and 100 Kdal, but only one, 257 Kdal, recognized by AD4 pool cells. This polyclonal serum reacted most strongly with the 200 Kdal band. The rabbit, infested with A. americanum adults, had polyclonal serum that recognized five bands, found in D. andersoni SGA with the same molecular weights recognized by the monoclonal antibodies. All five of these bands were recognized by the BG8 pool antibody: 300, 257, 200, 116,

and 100 Kdal; while, two, 257 and 234 were also recognized by AD4 pool antibody. The polyclonal rabbit serum strongly recognized the 257 and 116 Kdal bands. Thus seven of the bands recognized by BG8 pool antibody were also recognized by polyclonal serum from infested rabbits. Five of these bands are cross reactive being recognized in *D. andersoni* SGA by rabbit sera after infestation. If these cross reactive bands are associated with resistance, BG8 pool cells are producing antibody which has very significant potential for affinity isolation of antigen components whether it is mono or polyclonal.

A number of approaches were used to purify monoclonal antibodies, but an optimal strategy remains to be developed. The method of Bruck et al. (1986) was found to be unsatisfactory for purifying monoclonal antibody from RPMI culture medium, but appeared to work for ascites. Ascites production was successful in that 60 ml were produced, but unsuccessful in that the specificity of the antibody changed from recognizing one 24 Kdal band to recognizing approximately seven weak diffuse bands. Underwood and Bean (1985) reported dramatic changes in three of 38 monoclonal antibodies in cross reactivity patterns following ascites passage. This evidently reflects loss or mutation of hybridoma genetic information.

## SUMMARY AND CONCLUSIONS

Ixodid ticks transmit a variety of pathogens: viruses, rickettsia, bacteria, and protozoa, which lead to a vast array of diseases in man and livestock. Not only do ticks act as vectors of pathogens, they also serve as reservoirs by transmitting pathogens to their offspring by transovarial transmission, which assures that progeny are infected and increases the probability of transmission and survival of the pathogen. Additionally ticks pass pathogens from one life cycle stage to the next through transtadial transmission which potentially increases the number of host species pathogens may be transmitted to by three host ticks. The economic impact of ticks and tickborne diseases on the livestock industry world wide is staggering.

Control of ticks with chemicals, acaracides, was initially very effective. Then in the mid 1930s resistance to the first acaracide, sodium arsenate, was noted in Africa and Australia. Since then it has become apparent that ticks develop resistance to acaracides in a very short period. This has discouraged the production and marketing of new acaracides which are very expensive. The rapid development of acaracide resistance points to the need for alternative methods of control. One proposed alternative method is immunological control, attained through the development and use of an anti-tick vaccine.

Cattle and laboratory animals actively acquire resistance through multiple infestations. This resistance, which has an immunological basis, involves both antibody and cell mediated mechanisms as well as accessory components such as complement and histamine. The mechanisms

of acquisition and expression have not been fully described for any tick-host species association. Immunization with crude, whole tick extracts have induced some degree of resistance.

The purpose of this research was to identify components of these extracts that were possibly associated with the development of resistance. Following identification, these components were to be partially purified and then tested by immunization trials to evaluate their ability to induce resistance. Cross reactivity and cross resistance were investigated, as use of cross reactive components would potentially broaden the coverage provided by a vaccine. Production of monoclonal antibodies reactive with tick antigens was also undertaken.

Ultrafiltration and Sephadex G-200 gel filtration chromatography of A. americanum whole ova extract yielded defined molecular weight range fractions, of a heterogeneous nature, which were evaluated for immunogenicity by skin testing and then for efficacy by immunization trials. Skin testing resulted in both immediate and delayed hypersensitivity reactions in previously exposed guinea pigs. Ultrafiltration 30-100 Kdal fraction and its gel filtration counterpart, peak 2, both contained components in the 53 to 67 Kdal range. Immunization trials demonstrated that these components induced a stage specific resistance.

This resistance was characterized by decreased viability of larvae molting to nymphs and was comparable to the level of acquired resistance attained by active infestation of the two exposure controls.

Immunization of guinea pigs with 27,000 x g supernatant, prepared from A. americanum gut antigen, induced a significant level of resistance to infestation of guinea pigs with adult A. americanum. This

resistance was characterized by decreased engorgement weights, decreased egg mass weights, and death of 50% of the engorged female A. americanum. Thus it is apparent that resistance can be induced by vaccination. By refining the vaccines to contain only components associated with resistance and determining the most effective route of administration, levels of resistance exceeding those attained by multiple infestations should be achievable.

Cross reactivity between D. andersoni and A. americanum was demonstrated by immunoblot analysis. Dot-ELISA titers confirmed the findings of other investigators that circulating antibody titer was not directly related to the degree of resistance expressed. Cross resistance was shown to be very strong, between D. andersoni and A. americanum, through sequential infestation of guinea pigs. Sixty-two percent of engorged female D. andersoni died and only 12.5% laid eggs after engorging on guinea pigs previously infested by A. americanum larvae or nymphs.

Hybridoma cell lines were developed that produce antibodies to D. andersoni ova extract and SGA. These cells have been frozen in liquid nitrogen for later use and further characterization of their immunoglobulins. Although all statistical criteria have not been fulfilled and only one cell line recognizes a single polypeptide band, all three are believed to be monoclonal. These should prove to be useful tools in the further quest for specific components to incorporate into a vaccine which will induce resistance to tick infestation.

**APPENDICES**

## APPENDIX I

### ABBREVIATIONS AND SYMBOLS

BSA	bovine serum albumin
C	degrees Centigrade
CBH	cutaneous basophil hypersensitivity
CCHF	Crimean-Congo hemorrhagic fever
CFA	complete Freund's adjuvant
cm	centimeters
cm <sup>2</sup>	square centimeters
CO <sub>2</sub>	carbon dioxide
Dot-ELISA	dot enzyme linked immunosorbent assay
ECFa	eosinophil chemotactic factor of anaphylaxis
ELISA	enzyme linked immunosorbent assay
g	grams
>	greater than
HRPO	horseradish peroxidase
ID	intradermal
IFA	incomplete Freund's adjuvant
IgG	immunoglobulin class G
IgG1	immunoglobulin class G subclass one
IgM	immunoglobulin class M
IM	intramuscular
IP	intraperitoneal
IV	intravenous
Kdal	kilodalton

Kg	kilogram
KLH	Keyhole-limpet hemocyanin
LC	Langerhans cells
M	molar
ma	milliamperes
mg	milligrams
mm	millimeters
NaCl	sodium chloride
ng	nanograms
nm	nanometers
NS-1	P3-NS1-Ag4-1 mouse myeloma cells
PBS	0.15 M phosphate buffered saline, pH 7.4
PCA	passive cutaneous anaphylaxis
PEG	polyethylene glycol
pg	picogram
psi	pounds per square inch
RPMI	Roswell Park Memorial Institute medium
RSSE-TBE	Russian spring summer encephalitis-tick-borne encephalitis
SGA	salivary gland antigen
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
T-cell	T-lymphocyte (thymus derived cell)
x g	times gravity

## APPENDIX II

### SOLUTIONS

1. Phosphate buffered saline (0.15 M, pH 7.2)

8.0 g        NaCl  
0.2 g        KCl  
1.15 g       Na<sub>2</sub> HPO<sub>4</sub>  
0.2 g        KH<sub>2</sub> PO<sub>4</sub>

Bring volume to 1.0 liter with distilled water.

2. White's solution

0.25 g       HgCl<sub>2</sub>  
6.5 g        NaCl  
1.25 ml      HCl  
250 ml       95% ethanol  
750 ml       distilled water

3. Brush border fragment isolation buffer

12.1 g       Tris  
500 ml       distilled water

Titrate pH to 7.2 with 1 M HCl (approximately 89 ml), then add  
54.65 g D-mannitol.

### ELECTROPHORESIS REAGENTS

4. Stock acrylamide / bis (30% Total, 2.67% Crosslinker)

29.2 g       acrylamide  
0.8 g        N',N'-bis-methylene-acrylamide

Bring volume to 100 ml with distilled water.

Filter and store at 4C in the dark (30 days maximum).

**Caution** - acrylamide is a neurotoxin.

## 5. Stock 1.5 M Tris-HCl, pH 8.8

18.15 g Tris base

50 ml distilled water

Adjust pH to 8.8 with 1N HCl.

Bring volume to 100 ml with distilled water.

## 6. Stock 0.5 M Tris-HCl, pH 6.8

3.0 g Tris base

50 ml distilled water

Adjust pH to 6.8 with 1N HCl.

Bring volume to 100 ml with distilled water.

## 7. 10% SDS (w/v)

10 g sodium dodecyl sulfate (lauryl sulfate)

Bring the volume to 100 ml and dissolve by stirring.

## 8. 10% ammonium persulfate (w/v)

Prepare fresh for each use by dissolving 100 mg ammonium persulfate in 0.9 ml distilled water.

## 9. Separating gel (12% gel, 0.375 M Tris, pH 8.8)

13.5 ml distilled water

10 ml stock 1.5 M Tris-HCl, pH 8.8

0.4 ml 10% SDS (w/v)

16 ml stock acrylamide/bis

0.1 ml 10% ammonium persulfate (w/v)

0.002 ml TEMED

## 10. Stacking gel (4% gel, 0.125 M Tris, pH 6.8)

6.1	ml	distilled water
2.5	ml	stock 0.5 M Tris-HCl, pH 6.8
0.1	ml	10% SDS (w/v)
1.3	ml	stock acrylamide/bis
0.05	ml	10% ammonium persulfate (w/v)
0.005	ml	TEMED

## 11. Sample buffer

4.0	ml	distilled water
1.0	ml	stock 0.5 M Tris-HCl, pH 6.8
0.8	ml	glycerol
1.6	ml	10% SDS (w/v)
0.4	ml	2-mercaptoethanol
0.2	ml	0.05% bromophenol blue

## 12 10x electrode buffer

30.3	g	Tris base
144.6	g	glycine
1.0	liter	distilled water

Dissolve completely by vigorous shaking. Store at 4 C.

## 13. Upper chamber buffer

50	ml	10x electrode buffer
450	ml	distilled water
0.5	g	SDS

## 14. Lower chamber buffer

444 ml 10x electrode buffer  
4 g SDS  
400 ml distilled water

## 17. Stock 10% gluteraldehyde (v/v)

10 ml 100% gluteraldehyde  
90 ml distilled water

## 18. 2.5% gluteraldehyde (v/v)

5 ml stock 10% gluteraldehyde  
95 ml distilled water

## 19. Dithiothreitol

5 micrograms dithiothreitol  
1 ml distilled water

## 20. 3% Sodium carbonate

3 g sodium carbonate  
97 ml distilled water

## 21. 0.03% Sodium carbonate

1 ml 3% sodium carbonate  
99 ml distilled water

## 22. Developer

100 ml 3% sodium carbonate  
50 microliters 37% formaldehyde

## 23. 2.3 M Citric acid

44.16 g citric acid  
100 ml distilled water

## 24. 0.1% Silver nitrate

0.1 g silver nitrate  
99.9 ml distilled water

## 25. Buffalo black protein stain

2.0 l distilled water  
160 ml 95% ethanol  
40 ml glacial acetic acid  
0.5 g Buffalo black

## 26. Destaining solution for protein stain

2.0 l distilled water  
160 ml 95% ethanol  
40 ml glacial acetic acid

## 27. Transfer buffer

3.03 g Tris base  
14.4 g glycine  
1.0 liter distilled water

## 28. Tris-saline, pH 7.4

9.0 g NaCl  
1.21 g Tris base  
500 ml distilled water  
8.5 ml 1N HCl

Adjust pH to 7.4 with either 1N HCl or 1N NaOH.

Bring volume to one liter with distilled water.

## 29. Tris saline with 0.05% Tween-20 (v/v)

500 ml Tris saline

0.25 ml Tween-20 (Sigma Chemical Co., St. Louis, MO)

## 30. 5% BLOTTO

500 ml Tris saline

25 g nonfat dry milk (Carnation)

## 31. 5% BLOTTO with Tween-20

500 ml Tris saline

25 g nonfat dry milk

0.25 ml Tween-20

## 32. Stock 4-chloro-1-naphthal solution

.06 g 4-chloro-1-naphthal

20 ml anhydrous methanol

## 33. Precipitable substrate

10 ml .02 M phosphate buffered saline, pH 7.4 with 0.05%  
Tween-20

5 microliters 30% hydrogen peroxide

2 ml stock 4-chloro-1-naphthal solution

Prepare fresh for each use.

TISSUE PROCESSING

## 34. Sodium phosphate buffer (0.2 M, pH 7.2)

Solution A:

27.8 g  $\text{NaH}_2\text{PO}_4$ 

1 liter distilled water

Solution B:

53.65 g  $\text{Na}_2\text{HPO}_4$

1 liter            distilled water

Mix 28 ml of solution A with 72 ml of solution B and dilute with 100 ml distilled water.

35. Karnovsky's fixative

4            g            paraformaldehyde

46          ml          0.2 M sodium phosphate buffer, pH 7.2

In a fume hood dissolve 4 g paraformaldehyde in 46 ml of boiling 0.2 M sodium phosphate buffer, pH 7.2. If necessary clarify with a few drops of 1N NaOH. Mix equal volumes of this 8% paraformaldehyde solution with 10 % gluteraldehyde (v/v) in distilled water. Store in a dark bottle at 4 C.

36. Geimsa stain (Wolbach's modification)

Stain solutin:

2.5    ml    Geimsa stock (Allied Corporation, Fisher Scientific, Orangeburg, NY)

Differentiation solution:

10     g     colophonium

100    ml    absolute ethanol

37. Sodium cacodylate buffer (0.2 M, pH 7.2)

42.8    g            sodium cacodylate

Bring volume to one liter with distilled water.

38. 2% Uranyl acetate

0.4     g            uranyl acetate

10      ml            distilled water

Mix 1:1 with absolute ethanol.

Filter before each use.

## 39. Lead citrate (0.018%)

Freshly boil approximately 40 ml distilled water.

Mix 0.06 g lead citrate in 20 ml of cooled, boiled water.

Add 12 drops of 5 M NaOH.

Bring volume to 30 ml with cooled, boiled water.

Centrifuge for 15 minutes at 1300 x g.

Pipet and use supernatant; discard precipitate.

CELL CULTURE

## 40. Turk's solution

1 ml glacial acetic acid

1 ml 1% aqueous gentian violet

Bring volume to 100 ml with distilled water.

Filter before use.

## 41. Trypan blue stain

0.2 g trypan blue

100 ml PBS

## 42. Growth medium

500 ml RPMI (Gibco Laboratories, Grand Island, NY)

10 ml penicillin-streptomycin, 10,000 units each.  
(Gibco Laboratories, Grand Island, NY)

5 ml sodium pyruvate

75 ml fetal calf serum

## 43. HAT medium

100 ml growth medium

2 ml HAT supplement (Sigma Chemical Co., St. Louis, MO)

## 44. HT medium

100 ml growth medium  
2 ml HT supplement (Sigma Chemical Co., St. Louis. MO)

## 45. Spleen collection medium

100 ml RPMI  
2 ml penicillin-streptomycin, 10,000 units each (Gibco  
Laboratories, Grand Island, NY)

IgG PURIFICATION

## 46. Stock 1 M Tris-HCl, pH "7.2"

30.27 g Tris base  
100 ml distilled water

Adjust pH to 7.2 at room temperature with 1N HCl.

Bring the volume to 250 ml.

## 47. Column buffer (20 mM Tris, pH 7.2, containing 25 mM NaCl)

1.46 g NaCl  
20 ml stock 1M Tris-HCl, pH 7.2

Bring volume to 1 liter with distilled water.

## 48. Acetate-NaCl-isopropanol solution, pH 3.0

2.89 ml 99% glacial acetic acid  
40.9 g NaCl  
200 ml isopropanol

## 49. Prewash buffer (0.5 M NaCl in 20 mM Tris, pH 7.2)

14.61 g NaCl  
5 ml stock 1M Tris-HCl, pH 7.2

Bring volume to 250 ml with distilled water.

## 50. Regeneration buffer (2M guanidine HCl)

19.11 g      guanidine HCl

2      ml      stock 1M Tris-HCl, pH 7.2

Bring volume to 100 ml with distilled water.

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