COMPARISON OF THE IMMUNOSUPPRESSIVE ABILITY OF SALIVARY GLAND EXTRACTS OF THE IXODID TICKS: *DERMACENTOR ANDERSONI*, *AMBLYOMMA AMERICANUM* AND *RHIPICEPHALUS SANGUINEUS*

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Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 1996

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ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my advisor, and mentor Dr. Stephen Wikel for his guidance, and support throughout this study. Without his patience, persistence and the challenges he offered me, I would not have made it this far. I also want to thank Dr. Rangappa Ramachandra, and Dr. Douglas Bergman for the valuable discussions we had, and their helpful suggestions. The participation and help of all my committee members including Dr. Kim Burnham, Dr. Katherine Kocan, Dr. John Sauer, and the head of our department Dr. Russell Wright is highly appreciated. I thank all my instructors in the department of Entomology, and in other departments who helped me to grow, and develop a true appreciation of the complexities of the scientific world.

I would like to extend my thanks to the laboratory technicians Marie Montelongo, Angie Koscelny, and Denise Dalton. Thanks are due to Jerry Bowman, and Gesegesu Yemane in the Tick-rearing Facility, and to Bruce Nance in Laboratory Animal Resources. I would again like to acknowledge Dr. Stephen Wikel, and the Entomology Department for providing the funding and facilities to support my research.

I give special thanks to my colleagues and friends who provided me with support, understanding and companionship, including, Swarnjit Singh Chohan, Joe Dubois, and Douglas Starchman in our lab, and Qing Xu , David Brigham, Ying Qian, and Kuichun

Zhu in neighboring labs. Special thanks go to my friend Darryl Forest who's company I enjoyed while he drove me with him to EFAW in the morning.

Thanks are due to Dr. Alan Bowman for his help and kindness during my first semester here. I appreciate the help of Edmond Bonjour and Richard Grantham in transferring the slides to film, and their help with software problems. I would also like to thank all the secretaries for their excellent work and patience, and I thank all the people in the Entomology department who helped me, that I have not mentioned. I also want to thank Regina Henry, and all the people in the International Student Services Office for their help. I also acknowledge the late Dr. Scott Fargo for his help. The news of his death was sad, and difficult to understand.

My deepest love goes out to my family in Jordan. I thank my father for encouraging me to succeed ever since my childhood. My deep appreciation goes to my mother for always loving me, and helping me to overcome the difficulties of life, and for coaxing me to persist and not give up when things are rough. I also wish to acknowledge my sisters, who I have deeply missed, and for their support and the faith they had in me that inspired me to endure all the difficulties I faced, and helped me to succeed.

I dedicate this thesis to the memory of my only brother Kamal, who died so suddenly and unexpectedly at the peak of his youth, so he never had a chance at happiness in this world. I never had the opportunity to say good-bye, but I hope he has finally found eternal peace in his world of constant torment.

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NOMENCLATURE

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
BSF-1	B-cell stimulatory factor-1
C3a	complement factor 3a
C3b	complement factor 3b
C5a	complement factor 5a
CAM	cell adhesion molecule
CBH	cutaneous basophil hypersensitivity
$CD4^+$	cells positive for cluster of differentiation designation 4 (helper T-lymphocyte)
$CD8^+$	cells positive for cluster of differentiation designation 8 (cytotoxic T- lymphocyte)
CD23	cluster of differentiation designation 23
Con A	concanavalin-A
CPM	counts per minute
DNA	deoxy-ribonucleic acid

ELISA	enzyme-linked immunosorbant assay
FBS	fetal bovine serum
Fc	crystallizable fragment of antibody
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-monocyte colony-stimulating factor
HI	heat-inactivated
Ia	immune-response associated class II MHC molecules
ICAM-1	intercellular adhesion molecule-1
IFN-γ	gamma interferon
IgA	immunoglobulin-A
IgE	immunoglobulin-E
IgG_1	immunoglobulin-G_1
IgM	immunoglobulin-M
IL-1	interleukin-1
IL-2	interleukin-2
IL-4	interleukin-4
IMPDH	inosine monophosphate dehydrogenase
K	killer cell
kDa	kilodaltons
LAK	lymphokine activated killer cell
LC	Langerhans cell
LPS	lipopolysaccharide

2ME	2-mercaptoethanol
MCGF-2	mast cell growth factor-2
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MPA	mycophenolic acid
NK	natural killer cell
PAF	platelet activating factor
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
PHA	phytohemaglutinin
PMN	polymorphonuclear leukocyte
SE	standard error
SGE	salivary gland extract
TCGF-2	T-cell growth factor-2
T_H1	T-helper lymphocyte subset-1
T_H2	T-helper lymphocyte subset-2
TNF-α	tumor necrosis factor-alpha

CHAPTER I

PERSPECTIVES AND OVERVIEW

Introduction

Ixodid, or hard, ticks are obligate hematophagous ectoparasitic arthropods, and they are important vectors of pathogens of humans, and other animal species (Balashov, 1972; Fishbein & Dennis, 1995). Ixodid ticks have three life stages (larva, nymph and adult). Their ability to live for several years off the host, their large bloodmeal size, and prolific egg production, contribute to their success as ectoparasites (Sonenshine, 1991). Selected examples of tick-transmitted diseases include viral diseases such as, Colorado tick fever, and tick-borne encephalitis (Hoogstraal, 1981); rickettsial diseases such as, Rocky Mountain spotted fever, (Hoogstraal, 1981; Walker & Fishbein, 1991), ehrlichiosis (Silberg *et al.*, 1993), and anaplasmosis (Dikmans, 1950); bacterial diseases such as, Lyme borreliosis, (Barbour, 1992) and tick-borne relapsing fevers (Hoogstraal, 1981); and protozoal diseases such as, babesioses, (Anderson, *et al.* 1991) and possibly a new species of intraerythrocytic protozoan (WA1) (Thomford *et al.*, 1994).

Many of the world's serious infectious diseases are transmitted to humans and domestic animals by blood sucking arthropods (Mantovani, 1992). The rapid changes in most environments of the world brought about by population explosion, and agricultural development, have lead to an increasing number, and variety of arthropod-borne diseases, including tick-borne infections of humans, domestic animals and wildlife on all continents, leading to an increasing impact on world economies (Telford, *et al.* 1991). Tick infestation

causes a great economic impact (McCosker, 1979), and global cost was estimated to be $7 billion per year by the Food and Agriculture Organization of the United Nations (1984). The effective control of the vectors is one of the key factors to controlling many of these arthropod-borne diseases (Laird, 1985). A variety of methods have been used to suppress vector populations (Dobrokhotov, 1991), including, chemical insecticides (World Health Organization, 1992), environmental management (World Health Organization, 1980), physical methods (World Health Organization, 1988), and biological methods of control (World Health Organization, 1991).

Because ticks and other vectors have developed resistance to many acaricides and insecticides, and because of the impact of these chemicals on the environment, (Kunz & Kemp, 1994; World Health Organization, 1992), other alternative approaches to control such as the genetic manipulation of insect vectors, and immunological control are being investigated (Crampton, 1994; Curtis, 1985; Stiller, 1990; Wikel, 1982a, 1988, 1996). Immunological control requires an understanding of host responses to infestation, and the identification and characterization of the specific tick molecules that may be used to devise vaccines. The tremendous advances in biotechnology and molecular biology in the past few years provide the tools for research, leading to a better understanding of vector-host-pathogen interactions, making the task of the development of more effective methods for tick control a definite possibility (Wikel *et al.*, 1994).

Complex interactions occur between ticks, their hosts and the pathogens that ticks transmit (Allen, 1994; Friedhoff, 1990). The host has evolved immune mechanisms that help it to resist both infestation by ticks, and infection by the pathogens that are

transmitted to it by ticks (Brossard *et al.*, 1991; Wikel 1982a; Wikel & Allen, 1976a, b, 1977; Willadsen, 1980). In addition, ticks have developed countermeasures to facilitate successful feeding in the presence of the host immune defenses (Wikel *et al.*, 1994). Tick salivary glands secrete an array of immunogens that stimulate host immune responses, and induce host resistance to infestation (Wikel, 1982a, 1988; Willadsen, 1980). In contrast, tick saliva also contains several pharmacological substances that facilitate feeding by their anti-hemostatic and anti-inflammatory actions (Ribeiro, 1987a, Ribeiro *et al.*, 1990).Tick salivary secretions can also modulate the immune response to the vector (Inokuma *et al.*, 1993; Kubes *et al.*, 1994; Ramachandra & Wikel, 1992; Urioste *et al.*, 1994; Wikel, 1982c), and enhance the transmission of tick-borne pathogens (Jones *et al.*, 1992; Shaw *et al.*, 1993).

Immune responses to certain tick salivary components may provide resistance to infection with pathogens transmitted by the tick. An example is the increased resistance to infection by the tick-borne pathogen *Francisella tularensis* that was observed in rabbit hosts hypersensitized to *D. andersoni* ticks (Bell *et al.*, 1979). The identification of the molecules responsible for this phenomenon, and its utilization in anti-tick vaccines, may provide better methods for the control of tick-borne pathogens.

The molecules and mechanisms involved in the immunosuppressive activity of arthropod salivary glands are still poorly defined. The immunosuppressive activity could be due to one molecule with various activities, or to several molecules with different activities. It has been suggested by several authors that prostaglandin E_2 (PGE_2) present in tick saliva mediates immunosuppression by ticks (Bowman *et al.*, 1993; Ribeiro, 1987a;

Ribeiro *et al.*, 1985a). Tick salivary gland proteins or peptides have been shown to possess immunosuppressive and pathogen-transmission-enhancing properties by several investigators (Bergman *et al.*, 1995; Urioste *et al.*, 1994). Recently, immunosuppressive activity in a protein fraction with a molecular weight of 36-43 kilodaltons (kDa) from *D. andersoni* salivary glands has been identified (Bergman *et al.*, 1995), which establishes that proteins definitely have a role in the ability of tick salivary products to modulate host immune responses.

There is a lack of studies that compare the differences in patterns of immunomodulation of host responses, in different tick species, during the course of engorgement. The exact nature, mode(s) of action, and distribution of tick salivary gland immunosuppressive molecule(s) in different tick species is not yet well defined. Ixodid tick species may vary in the way they suppress the immune response of the host. The determination of the differences in the immunosuppressive ability among various ixodid tick species, is an essential prerequisite to the characterization of the chemical nature of these molecules, and to the examination of their mechanisms of action. Understanding how ticks modulate host immune responses is important for the development of efficient methods of control of ticks, and tick-borne pathogens. For an up-to-date review covering the various aspects of host immunity, including acquired resistance, pharmacological properties of tick saliva, tick modulation of host immune function, and anti-tick vaccines see (Wikel, 1996).

This dissertation is concerned with the comparison of the immunosuppressive abilities of the ixodid ticks *D. andersoni*, *A. americanum*, and *R. sanguineus*. A review of

the literature follows this introduction. The topics covered in this review include, host immunity to ticks, tick countermeasures to host defenses, host immune response modulation by arthropods, and a statement of hypothesis and objectives.

Host Immunity to Ticks

Immune Responses to Foreign Antigens

To better understand the immune response to ticks, a brief overview of the general immune responses to a foreign antigen, is provided before discussing the specifics of immune responses against ticks. Immune responses can be divided into nonspecific (innate immunity), and specific or (acquired immunity). The nonspecific mechanisms of innate immunity allow the body to detect and destroy most microorganisms that are encountered daily in the life of a healthy individual, and to clear foreign antigens from host tissues. Epithelial tissues protect body surfaces by providing a physical barrier between the external world containing pathogens, and the internal environment (Lehrer *et al.*, 1993). The alternative pathway of complement activation, which can proceed on some microbial surfaces, in the absence of specific antibody also provides a non-adaptive first line of defense against many microorganisms (Pangburn, 1986). Macrophages mature continuously from circulating monocytes and migrate into tissues throughout the body. These phagocytic cells have receptors for the Fc portion of certain immunoglobulin molecules, and receptors for complement components by which they can engulf opsonized particles. Macrophages also have receptors for various microbial constituents such as receptors for lipopolysaccharide (Wright, 1991). Macrophages also secrete cytokines due

to antigens binding to the same receptors used for engulfment. Cytokines are important in the next phase of defense, which consists of a series of induced but non-adaptive responses. The same receptors discussed above also play an important role in antigen uptake, processing, and the induction of co-stimulatory activity on macrophages, and thus in the induction of the adaptive immune response.

Cytokines (lymphokines and monokines) are soluble mediators that not only play a key role in the orchestration of immune responses , hematopoiesis, wound healing and inflammation, (Mantovani & Dejana, 1989; Miller and Krangel, 1992) but are also involved in normal physiological functions such as osteopoiesis (Lorenzo, 1991). Cytokines are proteins which are secreted by a wide variety of cells (Warrens & Lechler, 1992). Cytokine production is regulated by various inducers, and they have a short action radius, thus their production is often maintained for prolonged periods of time (Vilcek & Le, 1991). These molecules act in a paracrine and/or autocrine manner and produce their actions by binding to specific cell surface receptors. Cytokine functions include the promotion of leukocyte differentiation, proliferation, and homing (O'Garra, 1989a, b). Cytokines also regulate the production, (Fiorentino *et al.*, 1989; Moore *et al.*, 1990) and function (Spits *et al.*, 1988) of one another. Although a far greater range of cells is affected by cytokine action than can be evaluated, cytokines can be useful indicators of macrophage and lymphocyte activity during an immune response (Vilcek & Le, 1991). In this study, the immunosuppressive ability of tick salivary gland extracts of three ixodid tick species are compared. The effect of salivary gland extracts on the modulation of the

macrophage cytokines TNF-α and IL-1 and the lymphocyte cytokines IL-2 and IL-4 are determined.

Macrophage Cytokine Functions (TNF-α, IL-1)

To understand the importance of these cytokines in the immune response, a brief review of the events that comprise an acquired immune response, following the invasion of host tissue by a foreign substance is given. Macrophages engulf foreign substances, such as microbes, or tick salivary proteins, and then process and present antigens to T- and B-lymphocytes (Liu & Janeway, 1991). This leads to the elaboration of the macrophage cytokines TNF-α (Beutler & Cerami, 1989), and IL-1 (Durum *et al.*, 1985). Both TNF-α and IL-1 are proinflammatory cytokines that communicate to somatic tissues the presence of an inflammatory stimulus, such as the saliva secreted into the skin by a feeding tick. Many of the activities of TNF-α are similar to those of IL-1, and both TNF-α and IL-1 can augment the capacity of monocytes to produce other inflammatory mediators such as prostaglandins, IL-6, and IL-8 (Beutler & Cerami, 1986; Van Damme & Billiau, 1987).

Tumor necrosis factor-α is produced mainly by mononuclear phagocytes, although it can also be produced by a wide range of other cells such as NK cells , T-lymphocytes, B- lymphocytes, thymocytes and keratinocytes (Beutler and Cerami, 1989; Dinarello, 1988a). Tumor necrosis factor-α acts on a wide range of cellular targets, and causes a large number of cellular changes (Beutler & Cerami, 1989). Some changes are associated with the synthesis of new proteins while others are associated with the suppression of protein synthesis (Dinarello, 1988a). Tumor necrosis factor-α shows rapid cytotoxicity for some transformed cell lines (Mannel *et al.*, 1980; Old, 1985). This phenomenon is the

basis of the biological assay for TNF-α (Issekutz & Bhimji, 1982; Koide & Steinman, 1987).

Tumor necrosis factor exerts many direct and indirect effects on endothelial cells, and thus modifies various systems such as coagulation, cell adhesion, chemotaxis, and it has other effects on the vasculature (Beutler & Grau, 1993). Tumor necrosis factor favors procoagulant activity and thrombosis, by promoting the elaboration of tissue factor, platelet activating factor, and von Willebrand factor, and down-regulating the expression of thrombomodulin, thus decreasing the anti-coagulant property of endothelial cell surfaces (Clauss *et al.*, 1992; Stern & Nowroth, 1986).

Various sets of cell adhesion molecules (CAM) are either up-regulated or expressed *de novo* on the surface of endothelial cells, thus mediating the adhesion of various leukocytes, and other blood elements such as platelets (Gamble *et al.*, 1985; Gamble *et al.*, 1992). Leukocytes can then be mobilized to combat infection in relevant portions of the circulatory tree, while platelets can impede blood flow, and foster coagulation.

Tumor necrosis factor-α stimulates the release of chemotactic factors, and can inhibit the migration of neutrophils and monocytes (Ming *et al.*, 1987; Salyer *et al.*, 1990). Tumor necrosis factor-α augments the production of colony stimulating factors such as granulocyte-monocyte colony-stimulating factor (GM-CSF) by a variety of cell types, thus TNF-α indirectly activates hematopoietic cells, and triggers the directed locomotion of leukocytes such as macrophages (Munker *et al.*, 1986).

Other effects of TNF-α include; the induction of dermal inflammation (Sharpe *et al.*, 1987); the enhancement of the release of lysozyme, hydrogen peroxide, and prostaglandin E_2, prostacyclin I_2, thromboxane A_2, nitric oxide, and endothelin (Dudek *et al.*, 1992; Klebanoff *et al.*, 1986); the augmentation of neutrophil margination and the activation of the anti-microbial activity of macrophages (Bermudez & Young, 1988), neutrophils (Djeu *et al.*, 1986; Fong *et al.*, 1990), and eosinophils (Silberstein & David, 1986); and, the modulation of B-lymphocyte and T-lymphocyte function (Cordingley *et al.*, 1988). Tumor necrosis factor-α induces the expression of class I and class II MHC molecules, which are required for the presentation of antigens to T-lymphocytes by antigen-presenting cells such as macrophages (Beutler & Cerami, 1989). Finally, the effects of TNF-α can be harmful, and TNF-α is also termed cachectin because of its catabolic character and involvement in the pathophysiology of septic shock and wasting (Tracey, 1992).

The activities of IL-1 are important for the initiation of an acquired immune response, and for the mediation of inflammation (Mizel, 1987; Oppenheim & Gery, 1982). Interleukin-1 is produced when monocytes and macrophages are activated, and an acquired immune response is initiated, such as when these cells are challenged with lipopolysaccharide (LPS) (Koide & Steinman, 1987). Interleukin-1 is also synthesized by many other cell types including endothelial cells, fibroblasts, epithelial cells, astrocytes, keratinocytes and osteoblasts (Dinarello, 1988a, b).

Interleukin-1 has several diverse biological activities, including hematopoietic, immunologic, proinflammatory, metabolic and physiologic properties. Interleukin-1 was

previously known by a variety of names due to its various activities, and these names include endogenous pyrogen (Atkins, 1960); leukocytic endogenous mediator (Merriman *et al.*, 1977); lymphocyte activating factor (Gery & Waksman, 1972); mononuclear cell factor (Krane *et al.*, 1985); catabolin (Saklatvala *et al.*, 1985); osteoclast activating factor; and hemopoietin-1 (Stanley *et al.*, 1986). Interleukin-1 stimulates the immune system by participating in the direct activation of lymphocytes, and by indirectly inducing the synthesis of molecules that in turn activate lymphocytes.

The most often described T-lymphocyte activating property of IL-1 is its ability to act in a co-stimulator assay with sub-optimal concentrations of antigens or mitogens, which is demonstrated both in T-lymphocyte cell lines, and freshly obtained thymocytes (Simic & Stosic, 1985). Interleukin-1 amplifies T-lymphocyte activation by inducing IL-2 and IL-2 receptor gene expression, thus stimulating the production of IL-2, and the expression of IL-2 receptors on T-lymphocytes (Larsson *et al.*, 1980; Smith *et al.*, 1980). Interleukin-1 is also a cofactor for the proliferation and differentiation of B-lymphocytes, (Hoffman, 1980; Howard & Paul, 1983). Interleukin-1 induces the production of interferons, colony stimulating factors, T- and B-lymphocyte growth and differentiation factors, and other cytokines, and works synergistically with them on hematopoiesis (Bagby, 1989; Fibbe & Falkenburg, 1990; Stanley *et al.*, 1986; Van Damme & Billiau, 1987).

Interleukin-1 has both systemic and local effects on the responses of endothelial and smooth muscle cells . The effects of IL-1 on vascular epithelium promote the containment of infection and localization of injury (Movat *et al.*, 1987). Cultured

endothelial cells exposed to IL-1 synthesize PGE_2, PGI_2, and platelet activating factor, which are potent vasodilators (Dejana *et al.*, 1987). Interleukin-1 orchestrates a cascade of cellular and biochemical events that lead to vascular congestion, clot formation, cellular infiltration, and endothelial leakage. Interleukin-1 alters the surface receptors of edothelial cells, causing leukocytes to adhere and migrate into the extravascular tissue, and induces endothelium to express increased procoagulant and plasminogen activator inhibitor activity (Bevilacqua *et al.*, 1984). Interleukin-1 not only attracts leukocytes into inflamed tissues, but also causes degranulation of basophils and eosinophils, stimulates thromboxane synthesis in macrophages and neutrophils, and potentiates the activation of neutrophils by chemoattractant peptides (Klempner *et al.*, 1978; Luger *et al.*, 1983; Merriman *et al.*, 1977).

T-Lymphocyte Cytokine Functions (IL-2, IL-4)

Cytokine profiles of T-lymphocytes are used to characterize the nature of an immune response, whether it is a dominantly cell-mediated T_H1-type response, or a dominantly antibody-mediated T_H2-type response (Mosmann and Coffman, 1993). Activated helper T-lymphocytes of the T_H1 subset secrete an array of cytokines such as IL-2 and IFN-γ, which induces T-helper lymphocytes to proliferate and secrete more cytokines (autocrine stimulation of T-lymphocyte growth), and also activates various cellular effector functions. Activated helper T-lymphocytes of the T_H2 subset secrete an array of cytokines such as IL-4 and IL-5 that activate antibody effector functions (Mosmann and Coffman, 1993).

Interleukin-2 is produced by helper T- lymphocytes following activation by IL-1 (from macrophages), and by an antigen presented by major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (Vilcek & Le, 1991). Activated T-lymphocytes produce IL-2, and simultaneously express the high-affinity IL-2 receptor. Interleukin-2 induces a cytokine cascade with increased production of tumor necrosis factors, interferons, and interleukins, and thus exerts multiple effects via specific receptors expressed on a wide variety of cells including T-helper, cytotoxic and suppresser lymphocyte activity as well as B-lymphocytes, natural killer (NK) cells and cytotoxic macrophages (Whittington & Faulds, 1993).

Interaction of IL-2 with the IL-2 receptor induces T-lymphocyte proliferation and differentiation, and the mitogenic effect of IL-2 on T-lymphocytes may depend on the presence of monocytes (Mookerjee & Pauly, 1989). A proliferative response to IL-2 has also been observed with activated B-lymphocytes (Panayotides *et al.*, 1986), and IL-2 is essential for both early and late stages of B-lymphocyte differentiation (Xia *et al.*, 1989). Large granular lymphocytes (a subset of NK cell precursors), respond to IL-2 by increased proliferation and the production of IFN-γ (Koizumi *et al.*, 1986). Interleukin-2 augments the chemotactic activity of macrophages and T-lymphocytes (Melillo *et al.*, 1992), and macrophages display a rapid and direct response to IL-2, with a marked increase in cytotoxic activity (Malkovsky' *et al.*, 1987). Interleukin-2 generates lymphokine-activated killer cells (LAK) cells from a heterogeneous population of lymphoid cells, which are non-T-, non-B-, 'null' lymphocytes capable of killing a wide variety of tumor cells without MHC restriction (Damle *et al.*, 1986).

Interleukin 4 (IL-4) previously known as B-cell stimulatory factor-1 (BSF-1) is produced mainly by activated T_H2 subset of $CD4^+$ T-lymphocytes (Mosmann and Coffman, 1993), and it is also produced by mast cells and bone marrow stromal cells. Interleukin-4 acts as a co-stimulant in the activation, proliferation and differentiation of antigen-specific B-lymphocytes (Stein *et al.*, 1986; Vitetta *et al.*, 1985). Resting B-lymphocytes treated with BSF-1 demonstrated an increase in the density of class II MHC molecules on their surface (Noelle *et al.*, 1984; Roehm *et al.*, 1984). Interleukin-4 promotes the secretion of IgG_1 and IgE by B-lymphocytes (Snapper *et al.*, 1988a, b; Vitetta *et al.*, 1985).

Interleukin-4 is a growth factor for T-lymphocytes (Hu-Li *et al.*, 1987) and T-lymphocyte cell lines (Mosmann *et al.*, 1986), and was thus previously named T-cell growth factor-2 (TCGF-2) because of this activity. Interleukin-4 may play an important role in cognate T-lymphocyte-B-lymphocyte interactions, where B-lymphocytes bind antigen to their receptors, process that antigen, and express it on their surface in association with class-II MHC molecules. T-lymphocytes recognize the resultant antigen-class-II molecular complex and are stimulated as a result of this interaction (Ashwell *et al.*, 1984). This would in turn stimulate T-lymphocytes to secrete IL-4, and lead to an increase in the expression of class-II MHC molecules by resting B-lymphocytes, thus enhancing their capacity to act as antigen-presenting cells (Matis *et al.*, 1983).

Interleukin-4 in combination with an optimal concentration of IL-3 enhances the growth of mast cells, giving it the previous designation mast cell growth factor-2 (MCGF-2) (Mosmann *et al.*, 1986; Schmitt *et al.*, 1986; Smith & Rennick, 1986). Macrophages are activated when treated with IL-4, and acquire increased cytotoxic capacity, and display

enhanced expression of class-II MHC molecules (Crawford *et al.*, 1987; Meltzer *et al.*, 1987; Zlotnick *et al.*, 1986). Interleukin-4 synergizes with granulocyte colony-stimulating factor (G-CSF) to cause the appearance of granulocytic colonies, it synergized with recombinant erythropoietin to cause colony formation by immature erythroid precursors, and it enhanced the capacity of erythropoietin to stimulate colony formation by immature erythroid precursors (Peschell *et al.*, 1987). Interleukin 4 synergizes with the co-stimulants IL-1, erythropoietin, and a factor promoting megakaryocyte activity in the formation of megakaryocytic colonies (Long & Shapiro, 1985).

Immunity to Ixodid Ticks

After this brief discussion of cytokine function, the various aspects of immunity to blood sucking arthropods (specifically ixodid ticks) will be discussed. Ticks influence host responses to infection with tick-borne pathogens, and the host-tick-pathogen relationship is immunologically complex. Tick-transmitted *Borrellia burgdorferi* differed from needle inoculated organisms, in the immune responses they elicited in hamsters (Roehrig *et al.*, 1992). Ticks are hematophagous ectoparasites that stimulate a variety of host immune responses (Willadsen, 1980; Wikel, 1982a, 1996; Wikel & Allen 1976a, 1976b, 1977). Host immune responses can result in a variety of effects on ticks including simple rejection, interference with feeding, reduction in engorgement weights, diminished egg production, decreased viability of eggs, and death of the ectoparasite on the host (Willadsen, 1980; Wikel, 1982a, 1996; Wikel & Allen, 1976a, 1976b, 1977). Host grooming is stimulated by the itch sensation that can result in part from the immunological

reactions of the host to tick antigens (Alexander, 1986). Host grooming helps to reduce tick burden (Bennett, 1969).

The genetic background of hosts, such as bovines has for a long time been observed to be an important factor in the development of acquired immunological resistance to ticks (de Castro & Newson, 1993; George *et al.*, 1985; Johnston & Bancroft, 1918; Strother *et al.*, 1974). Friesian cattle carry higher numbers of *Amblyomma variegatum* ticks, and are more susceptible to dermatophilosis than N'Damas cattle (Koney *et al.*, 1994). *In vitro* lymphocyte proliferative responses of tick-infested Friesian and Zebu cattle to Con A were inhibited compared with lymphocytes from tick-free cattle, while N'Damas cattle lymphocyte responses were similar to those of tick-free Zebu types (Koney *et al.*, 1994). *Bos indicus* cattle have heightened immune responsiveness upon exposure to tick-salivary gland immunogens, when compared with *Bos taurus* cattle (George *et al.*, 1985; Ramachandra & Wikel, 1995; Wikel & Osburn, 1982). *In vitro* proliferative responses of *Bos indicus* peripheral blood lymphocytes to the T-lymphocyte mitogen Con A, or to the B-lymphocyte mitogen LPS, were significantly elevated over those of *Bos taurus* cells during exposure to SGE prepared from ticks on days zero to four of engorgement. *Bos indicus* peripheral blood mononuclear macrophages also produced more IL-1 than similar cells of *Bos taurus* origin either in the presence or absence of LPS.

Host resistance to tick infestation involves non-specific and specific immune responses including cell-mediated cutaneous basophil hypersensitivity reactions, complement-dependent immune effector mechanisms, homocytotropic antibodies and

circulating immunoglobulins, antigen-presenting cells, T-lymphocytes, cytokines, and other bioreactive molecules of the immune/inflammatory response (Brossard *et al.*, 1991; Wikel 1982a, 1996; Wikel & Allen, 1976a, 1976b, 1977; Willadsen, 1980).

Female ixodid ticks insert their mouthparts into the host skin, and remain attached for several days while they imbibe blood, and other exudates from a pool that forms around the mouthparts (Trager, 1939). Tick saliva has immunogenic properties which are potentially detrimental to the tick's feeding success, and the long period of attachment allows the development of host inflammatory reactions (Binnington & Kemp, 1980; Brossard *et al.*, 1991; Wikel, 1982a, 1984, 1988, 1996; Wikel & Allen, 1982; Wikel & Whelen, 1986). The salivary glands of female ixodid ticks undergo substantial growth and differentiation during feeding, and materials are accumulated, and depleted in specific cells of the multicellular, multialveolar tissue (Binnington, 1978; Sauer *et al.*, 1995; Walker *et al.*, 1985). Tick salivary gland and saliva proteins change their composition during feeding thus, the host is exposed to a changing array of immunogens during the course of tick engorgement (Gordon & Allen, 1987; McSwain *et al.*, 1982; Shapiro, *et al.* 1986). Antigenic changes in salivary glands have been monitored during feeding as a function of increase in tick weight, or the number of days after the start of feeding for female *A. americanum* (McSwain *et al.*, 1982), *Rhipicephalus appendiculatus*, (Shapiro, *et al.* 1986), and *D. andersoni* ticks (Gordon & Allen, 1987). Salivary gland extracts obtained from ticks at different times throughout the feeding process show differences both in their total antigenic content and makeup, thus the amount and number of salivary gland proteins of females change during the course of tick feeding.

The first attempt to characterize acquired resistance to ticks showed that guinea pigs could develop resistance after being infested for the first time with *D. variabilis* larvae (Trager, 1939). Langerhans cells are macrophage-like cells in the skin that are important for the development of acquired resistance to ticks (Allen *et al.*, 1979b; Nithiuthai & Allen, 1984a, b, c; Nithiuthai & Allen, 1985). Langerhans cells in the epidermis process immunogenic molecules (Streilein *et al.*, 1990), and migrate to the draining lymph nodes (Steinman, 1993), and present these molecules to antigen-specific T-lymphocytes in the context of class II MHC molecules on the surface of the antigen-presenting cells (Liu & Janeway, 1991). The treatment of guinea pigs with short-wavelength ultraviolet radiation prior to initial tick infestation, depleted Langerhans cells in the epidermis, and caused a reduction in the ability to acquire tick resistance (Nithiuthai & Allen, 1984b). Ultraviolet radiation treatment of tick-resistant animals before a challenge infestation reduced their ability to express anti-tick immunity (Nithiuthai & Allen, 1984c). During primary infestations, the number of Langerhans cells decreased in the epidermis (Nithiuthai & Allen, 1984a). Langerhans cells (LC) trap and process tick salivary immunogens (Allen *et al.*, 1979a, b), then migrate to the draining lymph node, and present them to immunocompetent lymphocytes (Nithiuthai & Allen, 1985; Streilein *et al.*, 1990). Minimal skin-reactivity is observed at tick attachment sites during the first exposure of a naive host to tick infestation, although both primary immunoglobulin (Trager, 1939; Willadsen, 1980; Wikel, 1982a; Wikel, 1984; Wikel & Allen, 1976b) and cell-mediated immune responses (Wikel *et al.*, 1978; Wikel & Allen, 1976a; George *et al.*, 1985) are induced.

Tick saliva induces the production of many antibodies with varying specificity (Shapiro *et al.*, 1986; Wikel, 1988), and the role of the various antibodies in immunity to ticks needs to be determined. Rabbits infested with *Hemaphysalis longicornis* ticks developed an IgG antibody to this tick species (Fujisaki, 1978). When the B-lymphocyte depleting compound cyclophosphamide was given to immune guinea pigs before an infestation with ticks, the expression of immunity was blocked (Wikel & Allen, 1976b). This provided indirect evidence for an antibody requirement in acquired tick immunity. The reduction of type I hypersensitivity (immediate antibody-mediated), and type IV hypersensitivity (delayed T-lymphocyte-mediated), with the administration of cyclosporin A, provided evidence for the involvement of T-lymphocyte-dependent responses in acquired resistance (Girardin & Brossard, 1989).

Increased levels of serum γ globulin of infested bovines caused a reduction in mean engorgement rates of *Boophilus decoloratus* (Rechav *et al.*, 1991). Certain salivary gland molecules, such as a 20 kDa molecule for *A. americanum* in guinea pigs (Brown & Askenase, 1986), and a 90 kDa molecule for *R. appendiculatus* in rabbits (Shapiro *et al.*, 1987), have been implicated in the induction of acquired resistance against ticks, and the roles of these molecules in the acquisition and expression of resistance should be further evaluated.

The antibody and cell-mediated responses (including lymphocyte, basophil, eosinophil and macrophage responses) generated in response to tick infestation eventually clear the tick antigens from the skin after the tick engorges, and detaches from the host

(Allen *et al.*, 1979a, b; Whelen *et al.*, 1986). Memory B- and T- lymphocytes specific for foreign antigens are usually produced after primary exposure (Vitetta *et al.*, 1991).

Repeated exposure to tick infestation may eventually lead to the development of resistance, and cutaneous inflammatory reactions are characterized by epidermal hyperplasia, edema, and the infiltration of granulocytes (Trager, 1939; Wikel & Allen. 1982). During the early phase of secondary infestation, when resistance is expressed, the number of Langerhans cells increases at tick-attachment sites (Nithiuthai & Allen, 1984a). More monocytes and also various granulocytes especially basophils, (Allen, 1973) are attracted to the tick attachment site by various chemotactic substances, that are released from cells (cytokines). A more rapid immune response resulting in higher antibody titers (mainly IgG) occurs with a second infestation, due to the presence of reactive antibodies and effector cells (Bowessidjaou *et al.*, 1977). The stimulation of memory B-lymphocytes and T-lymphocytes should lead to a maximal response in a few days (Gray, 1992; Vitetta *et al.*, 1991).

Acanthosis, intercellular edema of the epidermis, and granulocyte infiltration of the epidermis and dermis (predominantly basophils), characterize the immune response at the tick attachment sites for laboratory animals expressing acquired resistance to ticks (Allen, 1973, 1989; Brossard & Fivaz, 1982; Brossard *et al.*, 1991; Wikel & Whelen, 1986). Studies supporting the importance of basophils in limiting tick feeding include, the reduction of the expression of acquired resistance due to the administration of anti-basophil serum (Brown *et al.*, 1982), and the ability of mast cell-deficient mice to acquire resistance to *D. variabilis* (den Hollander & Allen, 1985b), which appears to be due to

the compensation of a basophil response for mast cell deficiency (Steeves & Allen, 1990). Basophil infiltration is a feature of what was previously termed 'Jones-Mote' delayed skin reactions in guinea pigs (Dvorak *et al.*, 1970; Richerson *et al.*, 1970). This reaction known as cutaneous basophil hypersensitivity (CBH) is a type of delayed hypersensitivity mediated by helper T-lymphocytes of the T_H1 subpopulation (Mosmann & Coffman, 1993).

As the tick introduces saliva into the feeding site for several days, circulating, and homocytotropic antibodies are produced. The levels of IgE and IgG_1 increase after tick feeding, which correlates with the immediate hypersensitivity component of resistance (Ushio *et al.*, 1993). Salivary antigens complex with homocytotropic antibodies, which are bound to Fc receptors on resident mast cells and incoming basophils (Brossard & Girardin, 1979; Whelen & Wikel, 1993; Wikel, 1996; Wikel & Whelen, 1986). This interaction of homocytotropic antibody and antigen results in the degranulation of the mast cells and basophils (Metzger & Kinet, 1988; Moore & Dannenberg, 1993; Wikel, 1996). The degranulation of basophils in the presence of salivary gland antigens becomes more rapid during repeated infestations (Brossard *et al.*, 1982). Mast cells and eosinophils are components of the cutaneous reactions at tick attachment sites on repeatedly infested animals, and are also important in establishing resistance (Brown *et al.*, 1982; den Hollander & Allen, 1985a). Mast cells degranulate at tick-attachment sites both during primary, and secondary tick infestations (Brossard and Fivaz, 1982). Tick gut cells of ticks that had fed on resistant hosts displayed signs of injury such as membrane damage, and basophil and eosinophil granules were observed within gut cells (Voss-McCowan, 1991).

The *in vivo* administration of cobra venom factor, a complement depletive factor, significantly inhibited the expression of tick resistance, indicating that complement contributes to expression of acquired resistance (Wikel & Allen, 1977). Complement-fixation by the classical pathway involves complexes of salivary gland antigens and antibodies, and complement components, all of which were found to be localized at the dermal-epidermal junction of tick-resistant guinea pigs (Allen *et al.*, 1979a). Guinea pigs that were C-4-deficient, thus totally deficient in the classical pathway of complement activation, could normally acquire and display tick resistance, indicating that the alternative pathway of complement activation is important in the expression of acquired immunity to ticks (Wikel, 1979). Levels of the complement component C3 were found to increase during tick infestations (Papatheodorou & Brossard, 1987). Activated complement components such as C5a are chemotactic for neutrophils, monocytes and basophils (Frank & Fries, 1989; Ward *et al.*, 1975). The anaphylatoxins C3a and C5a cause further degranulation of mast cell and basophils, and release of eosinophil chemotactic factors and vasoactive mediators such as histamine (Frank & Fries, 1989).

The degranulation of the mast cells, basophils, and eosinophils leads to the subsequent release of bioreactive molecules such as histamine, prostaglandins, and leukotrienes (Metzger & Kinet, 1988; Moore & Dannenberg, 1993). These vasoactive substances increase vascular permeability, leading to the accumulation of cells, complement and immunoglobulins at the bite site, and other endothelial changes (Bach, 1982). Animals showing acquired resistance had a higher histamine content at tick-attachment sites when compared to susceptible animals (Willadsen *et al.*, 1979; Wikel,

1982b). Histamine can affect feeding ticks directly (*in vivo* or *in vitro*) by reducing salivation and engorgement behavior, and stimulating detachment (Kemp & Bourne, 1980; Paine *et al.*, 1983), or indirectly by inducing grooming activity leading to the removal of ticks (Allen, 1987). The expression of resistance was reduced due to the administration of histamine receptor antagonists to tick-resistant animals (Brossard, 1982; Wikel, 1982b).

BALB/c mice were observed to acquire resistance after repeated infestations with *D. variabilis* larvae (den Hollander & Allen, 1985a). BALB/c mice did not acquire resistance to *Ixodes ricinus* nymphs, despite the development of cutaneous immediate- and delayed-type hypersensitivity reactions when they were reinfested (Mbow *et al.*, 1994a). When examining the skin of *Ixodes ricinus*-infested BALB/c mice using *in situ* hybridization for mRNA expression of various cytokines, and at the protein level by immunostaining with antibodies, the pro-inflammatory cytokines IL-1, and TNF-α, were detected in epidermal keratinocytes, dermal dendritic cells, and mononuclear cells (Mbow *et al.*, 1994b). The skin was also infiltrated with CD4+ and CD8+ T-lymphocytes and ICAM-1 and Ia molecules were expressed in the skin lesion (Mbow *et al.*, 1994b). A stronger expression of IFN-γ and IL-2 than of IL-4 mRNA was observed in infiltrating cells (Mbow *et al.*, 1994c), and the expression of IFN-γ and IL-2 mRNA increased in subsequent infestations, while the expression of IL-4 mRNA decreased. Although this indicates the presence of a cutaneous T_H1 response elicited by tick immunogens, it is important to note that these responses were not associated with a protective immune response in BALB/c mice against *I. ricinus* nymphal ticks infestations (Mbow *et al.*, 1994c). Studies of this type that compare animals that have developed resistance to ticks

to those that are not resistant, would provide a more clear picture of the cytokine patterns associated with a protective anti-tick immune response.

The continuous exposure of the tick to the host hemostatic, and inflammatory events, and immune response during feeding can lead to the restriction of blood flow to the tick attachment site, and can interfere with the successful engorgement of the tick, and may also hinder the ticks ability to transmit pathogens. Unsurprisingly, the tick has developed countermeasures including anti-hemostatic, anti-inflammatory and immunosuppressive abilities, that allow it to feed successfully despite the host immune defenses.

Tick Countermeasures to Host Defenses

Parasites have evolved numerous ways to survive in the presence of the immune responses of their hosts (Binaghi, 1993; Dessaint & Capron, 1993; Mitchell, 1991). Premunition or concomitant immunity (resistance to reinfection in hosts that are already infected) is common in helminth and protozoal infections where residing parasites survive, and escape elimination by the host immune response (Porter & Knight, 1974). Parasites survive host immune responses by multiplying faster than the host immune defenses can eliminate them, hiding from the immune response in sites not susceptible to attack, modifying surface molecular structure to look like host antigens, antigenic variation, rapid replacement of exposed immunogens, masking parasite antigens with host molecules, or impairing host immunity (Dessaint & Capron, 1993). Endoparasites can suppress host immunity by altering host cytokine production, antibody production, and cell-mediated

responses (Dessaint & Capron, 1993; Marrack & Kappler, 1994). Parasites can have various effects on the vectors that carry them. An example is *Trypanosoma rangeli*, that decreases the anti-hemostatic components, and blood vessel locating ability of its vector *Rhodnius prolixus* (Garcia *et al.*, 1994). This enhances the possibility of intradermal inoculation of the parasites into the mammalian host.

Several arthropod species utilize anti-hemostatic and anti-inflammatory strategies to aid in acquisition of a blood meal. The probing behavior of mosquitoes, and the time it takes to locate a blood vessel, and begin feeding differs from species to species. The less time it takes a mosquito to successfully obtain a blood meal, the better its chances of escaping before being removed by the host. *Anopheles stephanasi* probing behavior during blood location was found to be quantitatively different than that of *Aedes aegypti*, (Li & Rossignol, 1992). These differences may be related to different levels in salivary apyrase (Ribeiro *et al.*, 1985b). Female *Anopheles albimanus* mosquito saliva contains a heme peroxidase that functions during blood finding and feeding by destroying hemostatically active biogenic amines released by the host during tissue destruction (Ribeiro & Nussenzveig, 1993). Saliva of the sandfly *Lutzomyia longipalpis* contains a potent vasodilator peptide "maxadilan", which could inhibit the host hemostatic defenses (Lerner *et al.*, 1990). Deerfly (*Chrysops* sp.) salivary gland extracts contain a potent protein that inhibits platelet aggregation by competitively inhibiting fibrinogen binding to the platelet fibrinogen receptor, glycoprotein IIb/IIIa, thus preventing hemostasis (Grevelink *et al.*, 1993). The salivary glands of the triatomine bug *Rhodnius prolixus* contain a nitrosyl-heme protein "nitrophorin", that scavenges histamine, and releases the vasodilatory and

anti-platelet compound nitric oxide, thus counteracting the host hemostatic response (Ribeiro & Walker, 1994).

Similarly, ticks have evolved various countermeasures to survive the hemostatic, inflammatory and immune assaults on its attempts to successfully feed. Tick saliva possesses anti-hemostatic, anti-inflammatory and immunosuppressive activities (Ribeiro, 1987a). Although hemostasis, inflammation and immune responses are interlinked, and cannot be totally separated, this section will be mainly concerned with anti-hemostatic and anti-inflammatory tick countermeasures which reduce ability of the host to impair tick engorgement. Immunosuppressive countermeasures will be treated in the following section.

Anti-hemostatic activities are due to the presence of various enzymes and pharmacological agents in tick saliva (Ribeiro, 1987a). Enzymatic activities found in tick saliva include: 1) a salivary apyrase, which inhibits platelet aggregation induced by adenosine diphosphate (ADP), collagen or platelet activating factor (PAF) (Ribeiro *et al.*, 1985a); 2) a kininase that counteracts pain potentiation and edema promoting effects of PGE_2 (which potentiates pain produced by bradykinin, and potentiates the edema caused by substances that increase vascular permeability) (Ribeiro *et al.*, 1985a); and, 3) a carboxypeptidase that has both anaphylatoxin and bradykinin inactivating activities (Ribeiro & Spielman, 1986). The reduction of pain caused by cutaneous irritation could limit grooming thus reducing the removal of feeding ticks by the host. Anti-coagulants have been found in salivary gland homogenates of several ixodid ticks (Binnington & Kemp, 1980; Kemp *et al.*, 1982). Anti-coagulants present in the salivary glands inhibit

coagulation factors V and VII, blocking both the intrinsic and extrinsic pathway of coagulation (Gordon & Allen, 1991). A tick salivary anti-coagulant molecule was purified from *R .appendiculatus* salivary glands that inhibits the activity of factors of the prothrombinase complex such as factor Xa (Limo *et al.*, 1991). This molecule is poorly immunogenic, which may help to circumvent its damage by host-protective immune responses (Limo *et al.*, 1993). Prostaglandins were found in saliva, or salivary gland homogenates of several tick species such as PGE_2 in *A. americanum* saliva (Ribeiro *et al.*, 1992). PGE_2 may promote feeding by increasing blood flow to the tick mouthparts (Binnington & Kemp, 1980), and minimizing the release of platelet-aggregating, mast cell-degranulating, edema-promoting, and vasoconstrictive factors (Champagne, 1994; Ribeiro *et al.*, 1985a). *Ixodes dammini* saliva contains prostacyclin which induces vasodilation, and blocks platelet aggregation (Ribeiro *et al.*, 1988). It should be noted that *Ixodes dammini* is synonymous with *Ixodes scapularis* (Oliver *et al.*, 1993).

Anti-inflammatory activities overlap with anti-hemostatic activities due to the multiple biological activities of salivary-gland factors. Tick salivary apyrase which inhibits platelet aggregation by breaking down adenosine triphosphate (ATP) and adenosine diphosphate (ADP), to adenosine monophosphate (AMP) and orthophosphate (Ribeiro, 1987a; Titus & Ribeiro, 1990), also prevents inflammatory processes stimulated by ATP including mast cell degranulation and neutrophil aggregation (Bloom *et al.*, 1970; Ford-Hutchinson, 1982; Ischikawa *et al.*, 1972). Tick saliva contains carboxypeptidase activity that inactivates anaphylatoxins, reducing their chemotactic effects on granulocytes and monocytes, inhibiting the degranulation of mast cell and basophils and the subsequent

release of vasoactive mediators such as histamine (Frank & Fries, 1989; Ward *et al.*, 1975; Ribeiro & Spielman, 1986). Histamine and serotonin reduce sucking and salivation behavior of *in vitro* fed *D. andersoni* (Allen & Kemp, 1982; Paine *et al.*, 1983). Prostacyclin activity in *I. dammini* saliva and PGE_2 activity in *A. americanum* saliva are hypothesized to inhibit mast cell degranulation at the feeding lesion (Ribeiro *et al.*, 1988; Ribeiro *et al.*, 1992). Tick salivary glands have anti-histamine activity, that prevents the increase in vascular permeability caused by histamine, thus reducing the accumulation of antibodies, and cells of the immune system at the bite site, and likely avoids the inhibitory action of histamine on neuroreceptors that control tick feeding behavior (Chinery, 1981; Chinery & Ayitey-Smith, 1977).

Host Immune Response Modulation by Arthropods

Reactivity to various insect bites such as bedbugs, mosquitoes and fleas can change after repeated exposure, and lead to a state of unresponsiveness (Wikel, 1982c, 1984, 1996; Wikel *et al.*, 1994). Salivary products of several blood-feeding arthropod species, have demonstrated an ability to suppress or modulate mammalian host hemostatic, inflammatory, and immune responses. Suppression of host-immune responses are not limited to long-term blood feeders such as ixodid ticks, but were also observed in short term blood-feeders such as biting flies. Species that have demonstrated immunosuppressive abilities include; sand flies (Theodos & Titus, 1993), blackflies (Cross *et al.*, 1993; 1994b), mosquitoes (Bissonnette *et al.*, 1993; Cross *et al.*, 1994a), deerflies (Grevelink *et al.*, 1993), triatomine bugs (Ribeiro & Walker, 1994), mites (Corbett *et al.*,

1975; Laltoo & Kind, 1979), and ticks (Inokuma *et al.*, 1993; Kubes *et al.*, 1994; Ramachandra & Wikel, 1992, 1995; Urioste *et al.*, 1994; Wikel, 1982c, 1985, 1996; Wikel *et al.*, 1994). This widespread existence of immunomodulation of the host, suggests that it might be a common feature among blood-feeding arthropods. Vector-mediated immunosuppression likely evolved as a strategy to circumvent the host immune defenses that threaten the survival of the arthropod. The close association of vectors with the pathogens they transmit, also lead to the enhancement of pathogen transmission due to immunosuppression of host-immune responses by the arthropod.

Salivary gland material from the sand fly *Lutzomyia longipalpis* inhibited the ability of macrophages to present leishmanial antigens to parasite-specific T-lymphocytes (Theodus & Titus, 1993). Salivary gland extracts of *Aedes aegypti* suppressed the release of the poinflammatory cytokine TNF-α (Bissonnette *et al.*, 1993) from mast cells. *Aedes aegypti* salivary gland extracts significantly suppressed the T_H1 cytokines IL-2 and IFN-γ, but did not significantly suppress the T_H2 cytokines IL-4 & IL-5, in response to stimulation with the T-lymphocyte mitogen Con A (Cross *et al.*, 1994a). In this study, the proliferation of spleen cells in response to IL-2 was markedly suppressed by prior exposure of cells to SGE, while the proliferative response to IL-4 was affected to a lesser extent. An interesting observation in this study, was that SGE did not affect cytokine production by antigen activated cells derived from mice primed with the T-lymphocyte-dependent antigen ovalbumin.

In vivo inoculation of salivary gland extracts of the black fly *Simulium vittatum* reduced the percentage of Ia^+ cells in spleen cell populations, but had no effect on cells

derived from regional lymph nodes or the skin (Cross *et al.*, 1993). In this study, *Simulium vittatum* SGE reduced both T and B-lymphocyte mitogenesis *in vitro* (but not *in vivo*). Antigen-specific production of the cytokines IL-5 and IL-10 *in vitro* by spleen cells from *in vivo* ovalbumin primed mice was reduced due to repeated injection with SGE of *S. vittatum* (Cross *et al.*, 1994b), while levels of IFN-γ, IL-2 and IL-4 were not affected. In this study, mice repeatedly injected with SGE prior to ovalbumin challenge had fewer circulating eosinophils than saline-injected mice, although the levels of serum immunoglobulin A (IgA) or immunoglobulin E (IgE) were not significantly affected. *Simulium vittatum* SGE suppressed the *in vitro* proliferation of naive spleen cells in response to both IL-2 and IL-4 (Cross *et al.*, 1994b). The suppression of the production of various cytokines has a profound effect on the immune response, including the impairment of the ability to activate immunocompentent cells, present antigens, attract and sequester cells to the affected site, and the release of various mediators from cells. The type of immune response can also be affected, as certain cytokines can inhibit the production of other cytokines.

Several ixodid tick species suppress the host immune system including *D. andersoni* (Wikel, 1982c, 1985; Ramachandra & Wikel, 1992); *Dermacentor reticulatus* (Kubes *et al.*, 1994); *Ixodes dammini* (Urioste *et al*, 1994); *Ixodes ricinus* (Borsky *et al.*, 1994); and *Boophilus microplus* (Inokuma *et al.*, 1993). Stable tick-host interactions involve adaptation of ticks to their hosts' inflammatory and immune reactions, thus preventing the full expression of immunity at the tick-feeding site, which results in feeding and reproductive success (Ribeiro *et al.* 1990). The immunosuppressive activities of tick-

salivary products often result in a delicate balance between reducing the host immune defenses enough to allow successful engorgement, and maintaining sufficient immunocompetence to allow the survival of the host (Wikel *et al.*, 1994; Wikel, 1996).Tick salivary gland products have several immunosuppressive activities including those that suppress the function of various immune cells including B-lymphocytes, T-lymphocytes, NK cells monocytes, and granulocytes, and can also affect the activity of complement. Although some immunosuppressive activities have been attributed to cytotoxic activity such as reduced antibody responses to BSA for rabbits infested with *R. appendiculatus* (Fivaz, 1989), immunosuppression by most tick species is not caused by cytotoxicity (Wikel, 1982c; Ramachandra & Wikel, 1992; Wikel *et al.*, 1994). Although the precise mode of modulation may vary between species, it appears to operate at the cellular level of the host immune system in many cases, mainly by interference with the production and/or action of certain cytokines (Bissonnette *et al.* 1993; Ramachandra & Wikel, 1992, 1995; Cross *et al.*, 1994b).

Tick-infested laboratory animals and cattle showed a reduction in antibody responses. A reduction of hemolytic plaque-forming cell immunoglobulin M (IgM) response to a thymic-dependent antigen was observed for guinea pigs infested with *D. andersoni* (Wikel, 1985). Similarly, rabbit antibody responses to bovine serum albumin were reduced due to infestation with *R. appendiculatus* (Fivaz, 1989). *Boophilus microplus*-infested cattle showed a diminished antibody response against ovalbumin (Inokuma *et al.*, 1993).

T-lymphocytes collected from infested laboratory animals and cattle had reduced *in vitro* responses to T-lymphocyte polyclonal activators such as the mitogens concanavalin-A (Con A) and phytohemagglutinin (PHA). *In vitro* responses of peripheral blood lymphocytes of *Bos taurus* cows and calves to PHA was suppressed after three or four *D. andersoni* infestations (Wikel & Osburn, 1982). *In vitro* responses of cells derived from guinea pigs infested with *D. andersoni* to the T-lymphocyte mitogens Con A and PHA, were significantly depressed, while reactivity to *E. coli* LPS was not altered (Wikel, 1982c). Rabbit *in vitro* responses to Con A were reduced after infestation with *Ixodes ricinus* (Schorderet & Brossard, 1993). Splenic T-lymphocyte blastogenesis was inhibited in response to stimulation with Con A or PHA due to incubation with *I. dammini* salivary gland extracts (Urioste *et al*, 1994). A reduction in the percentage of T-lymphocytes was observed in cattle infested with *B. microplus*, and peripheral blood lymphocytes collected from tick-infested hosts had a reduced response to PHA, when compared to tick-free animals (Inokuma *et al*., 1993).

The *in vitro* reactivity of normal T-lymphocytes collected from tick-free laboratory animals and cattle to T-lymphocyte mitogens were suppressed due to incubation with tick salivary gland extracts or saliva. Ramachandra & Wikel (1992) have shown that crude salivary gland extracts of *D. andersoni* suppress normal murine lymphocyte proliferative responses to the T-lymphocyte mitogen Con A, but enhanced B-lymphocyte responsiveness to LPS. Similarly, *D. andersoni* salivary gland extracts suppressed the *in vitro* response of peripheral blood lymphocytes collected from pure breed *Bos indicus* and *Bos taurus*, but enhanced the response to LPS (Ramachandra & Wikel, 1995). *In vitro*

responses of normal bovine T-lymphocytes to PHA was reduced due to incubation with *B. microplus* saliva (Inokuma *et al.*, 1993).

Ixodes dammini saliva has a neutrophil-inhibiting function which inhibits neutrophil aggregation, granule enzyme function, super-oxide secretion and phagocytosis (Ribeiro *et al.* 1990). Nitric oxide production by macrophages stimulated with LPS was suppressed by *I. dammini* saliva (Urioste *et al*, 1994). *In vitro* NK cell activity of cells collected from healthy human donors was decreased due to incubation with *D. reticulatus* salivary gland extracts (Kubes *et al.*, 1994). Natural killer cells can lyse cancerous tumors, and virus infected cells without the requirement of MHC-restriction (Trinchieri, 1989), and the reduction of their function could impair the host immune defenses against tick-borne pathogens.

Complement activity is affected by tick salivary products. *Ixodes dammini* saliva contains an anti-complement activity that inactivates complement anaphylatoxins (Ribeiro & Spielman, 1986), and inhibits the deposition of C3b to activating surfaces, and the release of C3a (Ribeiro, 1987a, b) .

In vitro cytokine production by normal cells of laboratory animals and cattle was suppressed due to incubation with tick salivary gland products, and suppression of monocyte, and lymphocyte cytokines was observed. Cytokines are important orchestraters of the regulation of immune and inflammatory responses (Arai *et al.*, 1990; Kroemer *et al.*, 1993). Salivary gland extracts prepared from *D. andersoni* females suppressed macrophage production of the cytokines IL-1 (89.9% to 61.6% on days zero to five of feeding), and TNF-α (62.5%-94.6% on days zero to nine of feeding) (Ramachandra &

Wikel , 1992). *In vitro* suppression of splenocyte elaboration of IL-2 (14.1%-31.9%) and IFN-γ (8.7%-57%) was also observed (Ramachandra & Wikel , 1992). *In vitro* responsiveness of murine splenocyte to Con A and PHA was suppressed in the presence of *I. dammini* saliva by 77.4%, indicating inhibition of IL-2 production (Urioste *et al*, 1994). The elaboration of IL-1 and TNF-α by *Bos indicus* and *Bos taurus* macrophages was suppressed to a similar degree by female *D. andersoni* salivary gland extracts (Ramachandra & Wikel, 1995).

Ticks have evolved various countermeasures that target the main elements involved in the development of acquired host immunity. Macrophage function is suppressed by tick salivary gland extracts (Ramachandra & Wikel, 1992; Ramachandra & Wikel, 1995). Macrophages are important in the initiation of immune responses, and function to process and present antigens, and regulate the functions of both T- and B-lymphocytes (Knight & Stagg, 1993; Liu & Janeway, 1991). T_H1 functions are also targeted by tick salivary gland extracts (Ramachandra & Wikel , 1992), leading to a suppression of delayed-type hypersensitivity responses (Cher & Mosmann, 1987). The suppression of T_H1 activity can interfere with the production of IL-2 and the subsequent expansion of antigen-specific T-lymphocyte clones (Minami *et al.*, 1993; Smith, 1993); and the differentiation of B-lymphocytes (Xia *et al.*, 1989). Suppression of the T_H1 cytokine IFN-γ can inhibit macrophage activation (Munoz-Fernandez *et al.*, 1992; Stout & Bottomly, 1989), and the enhancement of NK cell activity (Trinchieri & Perussia, 1985; Trinchieri, 1989).

The influence of arthropod vectors on host responses to infection with an arthropod-borne pathogen reflects the complexity of the immunological interactions that are involved in the host-vector-pathogen interface. Ticks are not simply crawling hypodermic needles and syringes of pathogens, but modulate host immune responses to allow them to successfully obtain a blood meal. Tick-borne pathogens benefit from their long evolutionary relationships with ticks, because host immunosuppression results in an enhanced transmission of pathogens to new hosts.

The saliva of several arthropod vector species has been shown to have a role in enhancing the transmission of disease causing agents. Sandfly salivary gland material enhances the infectivity of *Leishmania* parasites, when the former is included in the infecting inoculum (Titus & Ribeiro, 1988, 1990). The exacerbative effect of salivary gland lysates varied in different mouse strains and applied to more than one *Leishmania* species and to more than one species of sandfly (Theodos *et al.*, 1991). Enhancement could not be obtained by preincubating parasites with salivary gland material and washing, thus this was not a direct effect on the parasite itself (Theodos & Titus, 1993). Salivary gland material from the sandfly *Lutzomyia longipalpis* inhibited the ability of macrophages to present leishmanial antigens to parasite-specific T-lymphocytes (Theodos & Titus, 1993).

Cupp *et al.*, (1994) suggested that salivary apyrase in African and New World vectors of *Plasmodium* species may be related to malaria transmission. The evolutionary trend of increased levels of the enzyme was associated with the speed of feeding

(decreased probing time) and expanded selection to include humans as hosts (Cupp *et al.*, 1994).

Tick suppression of immune responses can facilitate the transmission of pathogens. Tick salivary gland extract enhanced the susceptibility of lymphocytes to infection by *Theileria parva* sporozoites *in vitro* (Shaw *et al.*, 1993). The suppression of the immune response by tick infestation may also lead to the development of severe infections of opportunistic organisms, an example is the occurrence of dermatophilosis in Friesian cattle naturally infected with *A. variegatum* (Koney *et al.*, 1994). Proliferative responses of lymphocytes from tick infested Friesian cattle in Con A stimulated cultures were depressed when compared to acaricide-treated cattle soon after the former became tick-infested (Koney *et al.*, 1994). The addition of autologous serum to the lymphocyte cultures suppressed the proliferative response to Con A especially in cultures derived from tick-infested cattle (Koney *et al.*, 1994). Lymphocytes from tick-free cattle to which serum from tick-infested cattle had been added also showed a depressed proliferative response to Con A indicating the presence of circulating immunosuppressive factors in the serum of tick-infested cattle (Koney *et al.*, 1994). Transmission of a virus from infected to uninfected *Rhipicephalus appendiculatus* ticks (feeding on an apparently non-viremic guinea pig host) was enhanced due to a saliva-activated transmission factor (Jones, *et al.*, 1990). Enhanced Thogoto virus transmission occurred when guinea pigs were inoculated with virus plus a salivary gland extract of uninfected, partially fed *R. appendiculatus* (Jones, *et al.*, 1990). Transmission of this virus was enhanced when hosts were injected with saliva or salivary gland extract collected from ticks which had fed for a period of four

to seven days, prior to the injection of a viral inoculum to the same site (Jones, *et al.* 1992). This observation indicates that possibly immunosuppressive factor present in the tick saliva could have facilitated the transmission of this pathogen by dampening the immune response of the host, rather than having a direct effect on the virus. An examination of the immunocompetence of the host, and its correlation with virus transmission enhancement would provide more complete evidence for the presence of immunosuppressive factors that enhance virus transmission.

Host acquired resistance induced through the feeding of pathogen-free ticks can impair subsequent pathogen transmission by the same tick species (Wikel, 1980). Rabbit hosts hypersensitized to pathogen-free *D. andersoni* ticks were significantly more resistant to infection by exposure to *Francisella tularensis*-infected ticks (Bell *et al.*, 1979). Host resistance to tick infestation inhibited the transmission of viruses to the host (Jones & Nuttall, 1990). Similarly, *Ixodes ricinus* ticks infected with *Borrelia burgdorferi* could not transmit the spirochetes to hosts that were previously exposed to pathogen-free ticks (Dizij *et al.*, 1994). The immune responses of a tick-resistant host against feeding ticks could lead to the formation of an environment that inhibits pathogen development at the tick attachment site, and/or the development of host effector elements that neutralize tick immunosuppressive factors, thus allowing the development of effective host immune responses against the pathogen. Host resistance if found in nature may seriously limit the spread of tick-borne pathogens. The factors that facilitate the blocking of pathogen transmission, and the specific mechanisms involved in this process require characterization. Titus & Ribeiro, (1990) proposed the approach of controlling ticks and

tick-borne pathogens by the development of a vaccine that neutralizes tick-salivary gland derived immunosuppressive factors. Such a vaccine may have the beneficial effect of effectively reducing pathogen transmission, in addition to reducing tick burdens.

The role of PGE_2 in the immunosuppression of host responses by tick salivary secretions has yet to be established. Immunosuppression of *in vitro* responses of bovine peripheral blood mononuclear cells to PHA, induced by *B. microplus* salivary gland products, was attributed to factors other than PGE_2 (Inokuma *et al.*, 1993). In a subsequent study, *B. microplus* saliva containing 1.3 mg protein/ml, and 33 ng PGE_2/ml was used to suppress PHA responses (Inokuma *et al.*, 1994). The half-life of PGE_2 in blood is less than one minute (Trang, 1980), and most of its activity may be lost during the lengthy procedures of capillary-tube collection of tick saliva. Although the immunosuppressive effect of standard PGE_2 differed from that of *B. microplus* saliva, these authors concluded that PGE_2 in *B. microplus* saliva was responsible for the suppression (Inokuma *et al.*, 1994). Prostaglandin E_2 has an inhibitory effect on the production of cytokines by T-lymphocytes of the T_H1 subset (Betz & Fox, 1991), and the importance of tick salivary PGE_2 as a potential immunosuppressant has been proposed by other investigators (Ribeiro, 1987a; Ribeiro *et al.*, 1985a). Studies that compare immunosuppressive properties of whole saliva, to saliva that has been depleted of PGE_2 may show the relative contribution of salivary PGE_2 and salivary proteins to the modulation of host immunity.

The modulation of host immunity by ticks is mediated at least in part by salivary gland derived proteins. Enhancement of virus transmission, which is likely due to

immunosuppressive activity of *R. appendiculatus*, as discussed earlier is lost when salivary gland extracts are incubated with proteases (Jones, *et al.*, 1990). The immunosuppressive effects of *Ixodes dammini* saliva resulted from a protein with a molecular weight of 5.0 kDa or greater (Urioste *et al.*, 1994). The first immunosuppressive tick salivary activity to be isolated, and identified was found in a polypeptide (or protein) fraction that was isolated from *D. andersoni* salivary gland extract by Bergman *et al.*, (1995). This fraction was in the molecular weight range of 36-43 kDa, and significantly suppressed the *in vitro* proliferative responses of normal murine splenocytes (T-lymphocytes) to Con A (Bergman *et al.*, 1995). Further studies on other tick species should help to identify more tick immunosuppressive molecules, and to determine their mechanisms of action.

The study of the interactions that occur between ticks, their hosts and tick-borne pathogens provides a better understanding of the biology of tick feeding, tick salivary gland function; transmission of tick-borne pathogens; and methods of effectively controlling ticks and tick-borne pathogens. Although many great advances have been made in the past decade (Wikel, 1996), a lot of work must still be done to better understand the immunological complexities of the host-tick-pathogen interface.

Hypothesis and Objectives

Hypothesis

Different species of female ixodid ticks have different patterns of modulation of host immune response(s), which reflect(s) differences in their feeding patterns; rates of engorgement, salivary gland weight, and salivary gland protein content.

Specific Objectives

I. To determine differences in feeding rates and changes in salivary glands by characterizing the variation in tick weights, salivary gland weights and protein content of salivary gland extracts (SGE) on day 0 to day 7 of feeding for unmated *D. andersoni*, *A. americanum* or *R. sanguineus* females.

II. Demonstrate differences in the *in vitro* suppression of normal murine macrophage and lymphocyte function by salivary gland extracts prepared on days 0 to 7 of feeding for *D. andersoni*, *A. americanum* and *R. sanguineus.*

Determine the following:

a. Elaboration of the macrophage cytokines; tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1), and the T lymphocyte cytokines interleukin-2 (IL-2) (a T_H1 cytokine), and interleukin-4 (IL-4) (a T_H2 cytokine); and,

b. *In vitro* lymphocyte blastogenesis in response to concanavalin A (Con A) or *Escherichia coli* lipopolysaccharide (LPS) in the presence of salivary gland extracts.

CHAPTER II

MATERIALS AND METHODS

Tick Biology Studies

Laboratory reared , adult female *D. andersoni*, *A. americanum* and *R. sanguineus* ticks used in this study, were obtained from the colony maintained by the tick rearing facility of the Department of Entomology, Oklahoma State University. Ticks were raised according to procedures described by Patrick & Hair, (1976), where larvae were raised on restrained rabbits, while nymphs and adult ticks were reared on restrained female sheep by a modification of the method of Gladney and Drummond, (1970). Ticks were held in chambers maintained at 27° C and a photo-period of 14:10 day: night. Ticks that were used in this study were kept for three to five months after dropping off as engorged nymphs, and thus were between one to three months old, after molting into adults.

Tick feeding was done during the summer months (May-August). For tick biology studies, two hundred and forty unmated adult female ticks of each ixodid species were used. Unmated female ticks were allowed to obtain a blood meal from a female sheep, that had not been used in a previous tick infestation experiment. Six tubular medical stockinette cells were attached with industrial adhesive on the shaved back of the host, and one hundred and twenty ticks were placed in each cell. Fifty male ticks were placed into each cell for 24 hours, to condition the feeding area. All male ticks were removed just before infestation with female ticks. When *R. sanguineus* tick feeding was done, around 500 *R. sanguineus* nymphs were placed in each cell containing *R. sanguineus* females, to

promote better attachment. The nymphs were left to engorge and drop off normally. Thirty ticks from each species were collected daily during the course of engorgement from days zero to seven of feeding. Ticks were washed with reverse osmosis treated water, and all visible debris was removed. Ticks were then washed with 3% hydrogen peroxide (H_2O_2), rinsed with distilled water and left to dry. For each tick species, three groups of ten ticks each were weighed, and salivary glands were dissected in sterile 0.15 M phosphate buffered saline (PBS): (2.45 g KH_2PO_4; 8.1 g $Na_2H\ PO_4$; 4.38 g NaCl in 1 liter of distilled water, pH 7.2). For each group of ten ticks, a sterile 1.9 ml polypropylene microcentrifuge tube with cap (Elkay, Boston, MA) containing 100 µl PBS was tared on an analytical balance (Mettler Instruments, Hightstown, NJ), and salivary glands were collected into the tube and weighed. Salivary glands were stored at -20 °C overnight or until used. Salivary glands were thawed at room temperature, and sonicated five times for ten seconds each in a 270 watt/45 kHz bath sonicator (Branson, Shelton, CT), with cooling on ice. Glands were frozen at -20 °C overnight, thawed again and the above sonication procedure was repeated. The sonicate was centrifuged at 15,000 ×g in an Eppendorf microcentrifuge (Brinkman Instruments, Hamburg, Germany) for twenty minutes. Supernatant volume of each salivary gland extract (SGE) was determined to the nearest 5 µl. The protein content of each group of ten glands was determined, using the Bradford dye-binding assay (Bradford, 1976; Simpson & Sonne, 1982), as described below.

Preparation of Salivary Gland Extracts for *In Vitro* Assays

To prepare SGE for lymphocyte blastogenesis assays, and cytokine assays, six hundred unmated adult female ticks were fed according to the procedure used for tick biology studies. Seventy five ticks were collected on each of days zero to seven of feeding. Ticks used for preparing day 0 SGE were dissected as flat ticks that were obtained from holding chambers. Two complete sets of day zero to seven SGE were prepared for each tick species, by feeding a set of ticks as above twice, and collecting their glands .

Ticks were surface sterilized similar to the procedure used by Ramachandra & Wikel (1992), by washing them in distilled water, 3% H_2O_2 (Diamond Products, Seffner, FL), 1% sodium hypochlorite "Clorox" (The Clorox Company, Oakland, CA), 4% benzalkonium chloride "Roccal", (National Laboratories, Montvale, NJ), 70% ethanol, sterile distilled water and sterile distilled water containing 100 units penicillin and 100 µg streptomycin/ml (GIBCO BRL, Life technologies Inc., Grand Island, NY). Ticks were dissected under sterile conditions in a laminar flow hood, and salivary glands were collected in 100 µl sterile serum-free RPMI-1640 medium containing 100 units penicillin and 100 µg streptomycin/ml (see media preparation).

Salivary glands were removed, and washed at least three times in serum-free medium, by carefully separating glands from all nervous tissue, reproductive tissue and tracheal elements with fine forceps, and transferring the glands to fresh sterile medium. Salivary glands collected on a certain day, were pooled in a 1.5 ml sterile microcentrifuge tube containing 100 µl of sterile serum-free RPMI-1640, containing 100 units penicillin

and 100 μg streptomycin/ml, and held on ice. Salivary glands were stored at -20°C until used.

Salivary glands were thawed at room temperature, sonicated, and protein content for SGE supernatant was determined using the Bradford dye-binding assay (Bradford, 1976; Simpson & Sonne, 1982). Salivary gland extracts and pellets were stored at -20°C. Since day zero salivary glands were expected to have a very low protein content, 150 ticks were dissected for that day. Salivary gland extracts used for lymphocyte proliferation and cytokine assays were subjected to freeze-thaw once after determination of protein content.

Determination of Protein Content of SGE

The protein content of salivary gland extracts was determined, using a microplate version of the Bradford dye-binding assay (Bradford, 1976; Simpson & Sonne, 1982). Doubling dilutions of SGE were prepared ranging from (1/10-1/80) for days zero to one, and (1/20-1/160) for days two to seven. Bovine serum albumin (BSA) (2 mg/ml stock), (Pierce, Rockford, IL), was diluted in PBS (0.05-0.35 mg/ml), and served as a standard. The blank used was PBS. Triplicate aliquots of the blank, BSA standard dilutions or SGE dilutions (20 μl per well) were added to 96-well flat bottom microtiter plates (Corning, New York, NY). Dye reagent (Biorad Laboratories, Hercules, CA), was diluted (1 part to 3.75 parts H_2O), and filtered with a 12.5 cm grade four filter paper (Whatman Laboratories, Hillsboro, OR). Diluted dye reagent (180 μl) was added to each well, and microtiter plates were agitated for ten minutes.

Absorbance was determined at a wavelength of 595 nm using an automated plate reader (Biotek Instruments, Winooski, VT). Absorbance (y) was plotted for each standard (x), and the protein concentration (μg/ μl) was determined for each sample.

The mean protein content for the SGE collected from one pair of glands was estimated by the formula:

$$M = P \times V/10$$

M is the mean protein content of SGE for one gland pair

P is the protein concentration (μg/ μl)

V is the volume of SGE

Media Preparation and Culture Conditions for Lymphocyte Blastogenesis and Cytokine Elaboration Assays

Cytokine assays, and lymphocyte blastogenesis assays, were used to test for immunosuppressive properties of SGE, on each day of feeding. RPMI-1640 with L-glutamine, MEM non-essential amino acids, and MEM vitamin solution was the basic medium used for culturing throughout this study. This medium was prepared by dissolving one envelope of powdered RPMI-1640 with L-glutamine (2 mM) (GIBCO BRL, Life Technologies Inc., Grand Island, NY), 2 g cell culture grade sodium bicarbonate (SIGMA chemical company, St. Louis, MO), 10 ml MEM Non-essential amino acids (100X) (GIBCO BRL, Life technologies Inc., Grand Island, NY), and 10 ml MEM vitamin solution (100X) (GIBCO BRL, Life technologies Inc., Grand Island, NY), and making the volume up to one liter with triple distilled water. The pH was adjusted to 7.1, and the

medium was sterilized by filtering through a 0.22 μm filter with a filling bell (Millipore Corporation, Bedford, MA) with positive pressure, under sterile conditions.

Eight different batches of FBS were tested for endotoxin lipopolysaccharide levels, the batch with the lowest cytotoxicity was chosen for use in lymphocyte proliferation and cytokine assays. Minimal endotoxin levels in the medium are essential to prevent background error with the TNF-α assay. To test for endotoxin levels, murine peritoneal macrophages obtained from BALB/c mice as described in the section for preparation of macrophage supernatants below, and were incubated with eight different media preparations, with 10% HI FBS using a different batch of FBS for each medium. Macrophage supernatants were tested for TNF-α production, by testing the cytotoxic effect on L929 cells as in the TNF-α assay section described below. The fetal bovine serum that was chosen for use in this study was obtained from Atlanta Biologicals, Norcross, GA (Cat. No. S11150, Lot No. 3008L). Fetal bovine serum was heat-inactivated (HI) at 56°C for thirty minutes before using in culture medium. The antibiotics penicillin (100 units/ml), and streptomycin (100 μg/ml) were added to all media before use.

The medium for induction of TNF-α production by macrophages, L929 cell cultures, TNF-α assays and IL-1 assays was supplemented with 5 % HI FBS containing 100 units penicillin and 100 μg streptomycin/ml. Serum-free medium containing 100 units penicillin and 100 μg streptomycin/ml was used for the induction of IL-1 production by macrophages.

The medium used for lymphocyte proliferation, IL-2 and IL-4 induction by splenocytes was supplemented with 10% HI FBS containing 100 units penicillin and 100 μg streptomycin/ml. Medium for thymocyte co-mitogenesis (IL-1 assay), was RPMI-1640 supplemented with 10% HI FBS, and 0.5 mM 2-mercaptoethanol (2-ME) (SIGMA chemical company, St. Louis, MO), containing 100 units penicillin and 100 μg streptomycin/ml.

The base medium for CTLL-2 and CT-4S cell lines consisted of RPMI-1640 (prepared as mentioned above) supplemented with 0.1 mM sodium pyruvate (GIBCO BRL, Life technologies Inc., Grand Island, NY), 10% HI FBS, and 0.5 mM 2-ME.

The medium used for CTLL-2 cell lines was completed by adding 10% IL-2 containing supernatant to the base medium. To prepare the IL-2 containing supernatant, three spleens were collected from Albino Wistar rats (Charles River, Wilmington, MA), under sterile conditions in a laminar flow cabinet. Splenocyte suspensions were prepared by rubbing between sterile frosted slides, and cell density was adjusted to 2×10^6 cells/ml in RPMI-1640 with 5% HI FBS, 0.05 mM 2-ME, 15 M HEPES, containing 100 units penicillin and 100 μg streptomycin/ml, and 5 μg/ml Con A (CalBiochem, La Jolla, CA). Cells were cultured in T-75 tissue culture flasks, in 250-500 ml volumes. After 48 hours, the supernatants were harvested by centrifugation at 1000 ×g and 4°C for 10 minutes. The supernatants were divided into 20 ml aliquots, and 0.4 g of alpha methyl mannoside was added to each tube, to bind unused Con A. The supernatant was stored at -20°C until used. Immediately before use, the supernatant was thawed and filter-sterilized by passing through a 0.22 μm syringe filter (Millipore Corporation, Bedford, MA).

The medium used for CT-4S cell lines was completed by adding 10% IL-4 containing supernatant derived from X-4G transformed cell lines (gift from Dr. Alan Frey, New York University Medical School, New York, NY) to the base medium. The supernatant was prepared by culturing the X-4G cells in medium supplemented with 5% FBS, 0.1 mM sodium pyruvate, and containing 100 units penicillin and 100 μg streptomycin/ml. The cells were split every three to four days and grown at 1-2 $\times$ 10^6 cells/ml. Supernatant was harvested from cells grown for three to four days. The supernatant was stored at -20°C until used. Immediately before use, the supernatant was thawed, and filter-sterilized by passing through a 0.22 μm syringe filter.

Maintenance of Cell Lines

L929 Cell Line

A master-seed was prepared for L929 cells by expanding cells and freezing in liquid nitrogen for later use. Cells were initially grown in sterile 25 cm^2 tissue culture-treated polystyrene flasks, (Corning Costar corporation, Cambridge, MA) and then sterile Falcon 75 cm^2 flasks (Becton Dickinson & Company, Lincoln Park, NJ) were used to expand cells quickly for master-seed preparation. To prepare the master seed, subconfluent cells were cultured in T-75 flasks and split twice per week at a ratio of 1:4. Spent medium was removed, and 6 ml trypsin (GIBCO BRL, Life technologies Inc., Grand Island, NY), diluted to 1X (0.25%) in serum-free RPMI-1640 was added. Cells were incubated for up to five minutes at 25°C, and observed under an inverted microscope. Detached cells were placed into a sterile 15 ml centrifuge tube with a plug

seal cap (Corning, New York, NY) and were centrifuged at 600 ×g for five minutes. The trypsin solution was removed, and the pellet was dislodged by firmly tapping the tube with the fingers. The cells were resuspended in RPMI-1640 with 5% HI FBS containing 100 units penicillin and 100 µg streptomycin/ml.

For cell freezing, a sample of L929 cells was diluted (1/10) in a trypan blue stain solution (Sigma Chemical Company, St. Louis, MO), (0.2% in PBS), and cells were counted in a Neubauer chamber (0.1 mm deep hemocytometer) (Fisher Scientific, Pittsburgh, PA). Cultures to be frozen had a viability in excess of 85%. Cells were centrifuged at 600 ×g for five minutes, and the pellets were cooled on ice for five minutes. Cells were resuspended at a concentration of 1×10^6 cells/ml in cold (4 ° C) RPMI-1640 with 10% FBS and 20% dimethyl sulfoxide (DMSO) (Sigma Chemical Company, St. Louis, MO). Aliquots (1 ml each) of the cell concentrate were transferred to sterile 1.5 ml cryopreservation vials (Nalgene Products, Rochester, NY). The vials were placed in the vapor of liquid nitrogen for two hours, and then submerged in liquid nitrogen.

To reculture, frozen ampoules were placed in lukewarm water (30 ° C), and an equal volume of cold culture medium was added to them in a 15 ml centrifuge tube with a plug seal cap. The cells were centrifuged at 600 ×g for five minutes, and pellets were resuspended in cold medium. The cells were transferred to a sterile 25 cm^2 tissue culture flask, and incubated at 37 ° C in a humidified incubator with 5% CO_2.

CTLL-2 Cell Line

These nonadherent cells were split every two days at a ratio of 1:4. The cells were centrifuged at 600 ×g for five minutes, and resuspended in 5 ml fresh medium/25 cm^2 flask. The cells were incubated at 37 ° C in a humidified incubator with 5% CO_2. Fresh complete media was prepared weekly.

CT-4S Cell Line

This adherent cell line was split once a week at a ratio of 1:5 into 25 cm^2 flasks. The cells were removed from the surface of the flask with a sterile cell scraper. The cells were centrifuged at 600 ×g for five minutes, and resuspended in 10 ml fresh medium/25 cm^2 flask. The cells were incubated at 37 ° C in a humidified incubator with 5% CO_2. Fresh complete media was prepared weekly.

Lymphocyte Blastogenesis Assays

The effect of SGE on blastogenesis of normal murine splenocytes in response to stimulation with the mitogen Con A (Calbiochem, La Jolla, CA), or to *Escherichia coli* lipopolysaccharide (LPS) (SIGMA Chemical Company, St. Louis MO) was determined. The methods of evaluation of the lymphocyte blastogenesis response to mitogen were according to those described by Greaves & Janossy (1972). Female BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) that were eight to ten weeks old were used in these studies. Mice were housed at 22 ° C, and fed a commercial diet, and water *ad libitum* in an approved facility (Laboratory Animal Resources, School of Veterinary Sciences, Oklahoma State University).

Protein concentrations of SGE, on each day of feeding (zero to seven) were determined for each of the three tick species by the Bradford dye-binding assay as described earlier. Serum-free RPMI-1640 containing 100 units penicillin and 100 μg streptomycin/ml was used as the blank. Three concentrations of SGE (20 μg/ml, 10 μg/ml, and 5 μg/ml), were prepared for each day of feeding for each tick species in serum-free RPMI-1640 containing 100 units penicillin and 100 μg streptomycin/ml.

Female BALB/c mice (eight to ten weeks old) were dissected, spleens were removed, and a single cell suspension was made in medium supplemented with 10% HI FBS, containing 100 units penicillin and 100 μg streptomycin/ml by mechanical disruption between two sterile frosted glass slides (Fisher Scientific, Pittsburgh, PA). Slides used for this purpose were precleaned by washing for one hour in running tap water. Spleen cells were washed three times with centrifugation at 600 ×*g* for five minutes between washes, and resuspended in RPMI-1640 with 10% HI FBS. A sample of spleen cells was diluted Turk's solution [0.01 g gentian violet (Mallinckrodt, Paris, KY), 3 ml glacial acetic acid, and 97 ml distilled water]. Turk's solution lyses red blood cells, so that they will not be counted. Ninety μl of Turk's solution were put into each of three wells of a 96 well round bottom plate plates (Becton Dickinson & Company, Lincoln Park, NJ), and 10 μl of cell suspension were serially transferred from each well to the next, with thorough mixing making dilutions of 1/10, 1/100 and 1/1000. Cells were counted using a hemocytometer. One hundred μl of SGE, or control and 50 μl of splenocyte cell suspension containing 5 x 10^5 cells, were placed into wells of sterile Falcon 96-well tissue culture plates (Becton Dickinson & Company, Lincoln Park, NJ). After two hours of culture, 50 μl of either Con

A or LPS (20 µg/ml in RPMI-1640 with 10% HI FBS), for a final concentration of 1 µg/well of mitogen (10 µg/ml), which is the optimal concentration for the activity of these mitogens (Ramachandra and Wikel, 1992) was added.

The positive control wells each consisted of 100 µl of medium, 50 µl of splenocyte cell suspension containing 5 x 10^5 cells, and either 50 µl Con A or LPS (20 µg/ml in RPMI-1640 with 10% HI FBS). Positive controls represented the maximal blastogenesis response to these mitogens. The negative control wells consisted of 150 µl of medium, and 50 µl of splenocyte cell suspension containing 5 x 10^5 cells.

Cells were cultured for 54 hours, before pulsing with 20 µl of a 50 µCi/ml solution of methyl-tritiated thymidine (Dupont NEN Products, Boston, MA), to make a final concentration of 1 µCi per well. Triplicate wells were made for each test, and a minimum of 6 wells was made for each control. Cultures were incubated at 37 °C in a humidified 5% CO_2 incubator (NAPCO, Precision Scientific, Chicago, IL). After 18 hours, cellular nucleic acids were collected on glass fiber filter strips (Brandel Laboratories, Gaithersburg, MD), using a cell harvester (Brandel Laboratories, Gaithersburg, MD). Incorporation of methyl-tritiated thymidine was determined by using an automated liquid scintillation spectrophotometer (Beckman Instruments, Fullerton, CA).

Preparation of Macrophage Supernatants, Induction of IL-1 and TNF-α Production for Macrophage Cytokine Elaboration Assays

Salivary gland extracts prepared on each day of feeding (zero through seven) for *D. andersoni*, *A. americanum* and *R. sanguineus*, were assayed for their effect on

elaboration of the cytokines TNF-α, and IL-1, by macrophages. Macrophage monolayers were prepared with peritoneal exudate cells of eight to ten week old female BALB/c mice (Adams, 1980; Weid *et al.*, 1986; Kiener *et al.*, 1988; Tesh & Morrison, 1988). Four days before cell collection, mice were intraperitonealy injected with 3 ml of 3% sterile Brewer's thioglycolate broth (DIFCO Laboratories, Detroit, Michigan) (3 g thioglycolate added to 100 ml distilled water, and autoclaved). Cells were collected by peritoneal lavage with sterile RPMI-1640, containing 100 units penicillin, and 100 μg streptomycin. Mice were euthanized by placing in a chamber containing diethyl-ether, pinned ventral side up to a dissecting board, and surface sterilized with 70% alcohol. Under sterile conditions, in a laminar flow cabinet, the skin was lifted with forceps, and 3 ml sterile RPMI-1640 with 5% FBS containing 100 units penicillin and 100 μg streptomycin/ml, was injected into the peritoneal cavity. The abdomen was gently tapped to suspend cells in the peritoneum. The skin layer was removed, by making an incision down the center of the abdomen. The muscle layer was clasped with forceps, and a small incision was made in the lower abdominal area. A sterile Pasteur pipette was inserted, and the abdominal cavity was gently lavaged several times, and the cell suspension was collected into a 15 ml centrifuge tube with a plug seal cap. Cells were washed once with centrifugation at 600 X *g* for 5 minutes, resuspended in RPMI-1640. A 10 μl sample of the cells was diluted (1/10, 1/100, 1/1000) in a trypan blue stain solution (0.2% in PBS), the viability was determined, and the cells were counted with a hemocytometer using a suitable dilution. Cells (viability >85%) were plated at 5×10^5 cells per well in 1 ml of RPMI-1640 with 5% FBS in 24-well Falcon tissue culture plates (Becton Dickinson & Company, Lincoln Park, NJ). Non-

adherent cells were removed after two hours of incubation, by washing twice with RPMI-1640 containing 100 units penicillin and 100 μg streptomycin/ml. The resulting macrophage monolayers were used to induce IL-1 and TNF-α production.

Macrophage monolayers were cultured by incubating at 37 ° C in a humidified incubator with 5% CO_2 in the presence of three concentrations of SGE (20 μg/ml , 10 μg/ml and 5 μg/ml), in 1 ml/well volume for two hours. Twenty μl/well of LPS (100 μg/ml) was added to give a final concentration of 2 μg/ml per well, and culture supernatants were harvested at 18 hours for TNF-α assay, and 48 hours for IL-1 assays.

The positive control wells consisted of macrophage monolayers, RPMI-1640 with 5% HI FBS containing 100 units penicillin and 100 μg streptomycin/ml (1 ml), and 20 μl/well of *E. coli* LPS (100 μg/ml). The positive controls represented the maximal production of TNF-α and IL-1. The negative control wells consisted of macrophage monolayers, and RPMI-1640 with 5% HI FBS containing 100 units penicillin and 100 μg streptomycin/ml (1 ml), cultured without LPS stimulation.

Supernatants were collected and stored at -20 ° C until used. The viability of the macrophage monolayers was checked by adding a drop of trypan blue stain solution (0.2% in PBS) to each well, and examining for any dye uptake under an inverted microscope.

TNF-α Assay (L929 Fibroblast Cell Cytotoxicity Assay)

TNF-α levels were determined by a cytotoxicity assay, using L929 murine fibroblast cells cultured in the presence of actinomycin-D (GIBCO BRL, Life technologies Inc., Grand Island, NY) (Issekutz & Bhimji, 1982; Koide & Steinman, 1987). Actinomycin-D (an inhibitor of transcription) intercalates DNA, preventing L929 cells from synthesizing protective proteins, making them more susceptible to lysis by TNF-α (Gadelle & Forterre, 1994). Murine fibroblast L929 cells were cultured by incubating at 37 ° C in a humidified incubator with 5% CO_2 in 75 cm^2 sterile tissue culture flasks until confluent as described in the section for cell line maintenance above. Trypsin (1X) (0.25% in serum-free RPMI-1640) was used to dislodge the cells from the flask surface as described earlier, and the cells were washed three times by centrifugation at 600 ×g. A sample of the cells was diluted in a trypan blue stain solution (0.2% in PBS), the viability was determined, and the cells were counted with a hemocytometer. L929 cells (viability >85%) were grown overnight by incubating at 37 ° C in a humidified incubator with 5% CO_2 in 96-well, flat bottom microtiter plates at a concentration of 2 x 10^4 cells in 100 µl/well. Culture medium was removed, and replaced with 100 µl of test, or control macrophage supernatant. Triplicate wells were prepared for each test, and a minimum of six wells were prepared for each control. Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere. Actinomycin D (GIBCO BRL, Life Technologies Inc., Grand Island, NY) (20 µl of a 5 µg/ml solution in serum-free RPMI-1640, was added to wells (i.e. 0.1 µg/well), and cultures were incubated for 24 hours.

The positive control wells consisted of L929 cell monolayers cultured with supernatants from LPS-stimulated macrophages (100 µl), and 20 µl of a 5 µg/ml actinomycin D solution. Lysis of L929 cells is maximal in the positive control due to the maximal production of TNF-α. The negative control wells consisted of L929 cell monolayers cultured with unstimulated macrophage supernatants (100 µl), and 20 µl of a 5 µg/ml actinomycin D solution. The negative control wells showed the magnitude of background endotoxin activity of FBS. The actinomycin-D control wells (to check activity), consisted of an L929 cell monolayer, RPMI-1640 with 5% FBS containing 100 units penicillin and 100 µg streptomycin/ml (100 µl), and 20 µl of a 5 µg/ml actinomycin D solution. The cells only control wells consisted of L929 cell monolayer, and RPMI-1640 with 5% FBS containing 100 units penicillin and 100 µg streptomycin/ml (100 µl), cultured without actinomycin-D to check their viability.

Culture supernatants were discarded, leaving surviving L929 cells adherent to the well bottoms. Plates were air-dried overnight, and the cells were stained for 10 minutes at room temperature with 100 µl/well of 0.5% gentian violet in 20% methanol. The plates were washed gently in a cold water bath under running water (reverse osmosis-treated), until the water ran clear. Excess water was removed from the wells by firmly flicking the plates, and gently tapping on paper towels. The plates were air-dried overnight, and stain was solubilized in Sorensen's buffer [6.1 ml of 0.1 M citric acid (Fluka BioChemica, Buchs, Switzerland), 3.9 ml of 0.1N HCl, and 10 ml of 95% ethanol]. Retained dye was quantitated by determining the optical density (O.D.) at 562 nm.

IL-1 Assay (C3H/HeJ Mouse Thymocyte Co-Mitogenesis Assay)

IL-1 levels were determined by evaluating the co-stimulatory effect of LPS-stimulated macrophage supernatants (containing IL-1), on the proliferative response of murine thymocytes when stimulated with the mitogen Con A (Gery *et al.*, 1972; Tesh & Morrison, 1988), at a final concentration of 1 μg/ml. Thymocytes were collected from five to six week old LPS-nonresponder C3H/HeJ mice (Jackson Laboratories, Bar Harbor, Maine), counted using Turk's solution (described above). Cell concentration was adjusted to 5 $x10^5$ cells/well in RPMI-1640 with 10% FBS, 0.05 mM 2-ME, and 20 μg/ml Con A. Fifty μl of test or control macrophage supernatants, and 50 μl of cell suspension were added to each well. The positive control wells consisted of cell suspension containing 20 μg/ml Con A (50 μl), and supernatants of LPS-stimulated macrophages (50 μl). The positive control wells showed the maximum co-mitogenic effect of IL-1 on Con A-stimulated thymocytes. The negative control wells consisted of cell suspension containing 20 μg/ml Con A (50 μl), and unstimulated macrophage supernatants (50 μl). The Con A control wells (to check its activity), consisted of cell suspension containing 20 μg/ml Con A (50 μl), and 50 μl of RPMI-1640 with 5% FBS, containing 100 units penicillin and 100 μg streptomycin/ml. The cells only control wells consisted of a suspension of unstimulated thymocytes prepared in RPMI-1640 with 10% FBS, and 0.05 mM 2-ME.

Triplicate wells were made for each test, and a minimum of six wells was made for each control. Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere for a total 72 hours. Six hours before termination of cultures, cells were pulsed with 20 μl of a 50 μCi/ml solution of methyl-tritiated thymidine, to make a final concentration of 1 μCi

per well. Cellular nucleic acids were collected on glass fiber filters, using a cell harvester. Incorporation of methyl-tritiated thymidine was determined by using an automated liquid scintillation spectrophotometer.

Preparation of Splenocyte Supernatants, Induction of IL-2 and IL-4 Production for Splenocyte Cytokine Elaboration Assays

Salivary gland extracts prepared on each day of feeding (0 through 7) were assayed for their effect on elaboration of splenocyte T-lymphocyte cytokines including, the T_H1 subset cytokine IL-2 and the T_H2 subset cytokine IL-4. Spleens were dissected from eight to ten week old BALB/c mice and placed into sterile RPMI-1640 with 10% HI containing 100 units penicillin and 100 µg streptomycin/ml. A single cell suspension was prepared by mechanical disruption in the medium between sterile, frosted glass slides. Cells were washed three times by centrifugation at 600 X *g* for five minutes each time, and resuspended in the Con A-free base medium used for culturing CTLL-2 or CT-4S cell lines.

A sample of spleen cells was diluted in Turk's solution (described above), and cells were counted with a hemocytometer. Splenocyte cell concentration was adjusted to 1 x 10^7 cells/ml. Splenocytes were cultured in the presence of three concentrations of SGE (20 µg/ml , 10 µg/ml, and 5 µg/ml) for two hours. Five hundred µl of SGE, or control, and 250 µl of a 1 x 10^7 cells/ml cell suspension (containing 2.5 x 10^6 splenocytes), were placed in each well of a 24-well plate. Concanavalin A (250 µl/well of a 20 µg/ml solution) was added to give a final concentration of 5 µg/ml per well.

Positive control wells consisted of 500 µl of RPMI-1640 with 10% FBS containing 100 units penicillin and 100 µg streptomycin/ml, a 1 x 10^7 cells/ml cell suspension containing 2.5 x 10^6 splenocytes (250 µl), and 250 µl of a 20 µg/ml Con A solution prepared in RPMI-1640 with 10% FBS containing 100 units penicillin and 100 µg streptomycin/ml, to give a final concentration of 5 µg/ml per well. The negative control wells consisted of 750 µl of RPMI-1640 with 10% FBS containing 100 units penicillin and 100 µg streptomycin/ml, and a 1 x 10^7 cells/ml cell suspension containing 2.5 x 10^6 splenocytes (250 µl).

Culture supernatants were harvested after 54 hours for both IL-2 assays, and IL-4 assays. Supernatants were stored at -20°C until used.

IL-2 Assay (CTLL-2 Proliferation)

IL-2 levels were determined by evaluating the proliferation of the IL-2-dependent CTLL-2 cell line (Hamblin & O'Garra, 1987). Proliferation was determined by measuring the incorporation of methyl-tritiated thymidine into cellular DNA. CTLL-2 cells were maintained in RPMI-1640 medium containing 10% HI FBS, and a 10% supplement of IL-2 containing supernatant derived Con A- stimulated rat splenocytes. Cells were washed twice in medium lacking Con A supernatant by centrifugation at 600 × *g* for five minutes, viability was determined, and cells were counted using a trypan blue stain solution (0.2% in PBS) before use in the assay. Cells (viability >85%) were resuspended at a concentration of 1 x 10^5 cells/ml in medium with 10% HI FBS, containing 100 units

penicillin and 100 μg streptomycin/ml. Fifty μl of test or control splenocyte supernatant, and 50 μl of CTLL-2 cell suspension were added to each well, and incubated for 18 hours.

The positive control wells consisted of Con A-stimulated splenocyte supernatant (50 μl), and CTLL-2 cell suspension (50 μl). The negative control wells consisted of unstimulated splenocyte supernatant (50 μl), and CTLL-2 cell suspension (50 μl). The IL-2 control wells (to check its effect on CTLL-2 proliferation) consisted of Con A-stimulated rat splenocyte supernatant (containing IL-2) (50 μl), and CTLL-2 cell suspension (50 μl). The cells only control wells (representing the effect of culturing CTLL-2 cells with IL-2- free medium), consisted of RPMI-1640 with 10% FBS containing 100 units penicillin and 100 μg streptomycin/ml (50 μl), and CTLL-2 cell suspension (50 μl).

Triplicate wells were prepared for each test, and a minimum of six wells were prepared for each control. Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere. Cells were pulsed with 20 μl of a 25 μCi/ml solution of methyl-tritiated thymidine, to achieve a final concentration of 0.5 μCi per well, and cultured for five hours. Incorporated label was harvested, and counted by use of a liquid scintillation spectrophotometer.

IL-4 Assay (CT-4S Proliferation)

IL-4 levels were determined by evaluating the proliferation of IL-4-dependent CT-4S cell line (Hu-Li *et al.*, 1989). Proliferation was determined by measuring the incorporation of methyl-tritiated thymidine into cellular DNA. CT-4S cells were

maintained in RPMI-1640 supplemented with 0.1 mM sodium pyruvate 10% HI FBS, and 0.5 mM 2-ME, containing 100 units penicillin and 100 μg streptomycin/ml, and a 10% supplement of IL-4 containing supernatant derived from confluent X-4G transformed cell line. Cells were washed twice in medium by centrifugation at 600 × *g* for five minutes, the viability was determined, and cells were counted using a trypan blue stain solution (0.2% in PBS) before use in the assay. Cells (viability >85%) were resuspended at a concentration of 5 x 10^4 cells/ml in IL-4-free medium (RPMI-1640 supplemented with 0.1 mM sodium pyruvate 10% HI FBS, and 0.5 mM 2-ME, containing 100 units penicillin and 100 μg streptomycin/ml). One hundred μl of test or control splenocyte supernatant, and 100 μl of a 5 x 10^4 cells/ml CT-4S cell suspension (5 x 10^3 cells/well) were added to each well of a 96-well plate, and incubated for 48 hours.

The positive control wells consisted of Con A-stimulated splenocyte supernatant (100 μl), and 100 μl of a 5 x 10^4 cells/ml CT-4S cell suspension (5 x 10^3 cells/well). The negative control wells consisted of unstimulated splenocyte supernatant (100 μl), and 100 μl of a 5 x 10^4 cells/ml CT-4S cell suspension (5 x 10^3 cells/well). The IL-4 control wells (to check its effect on CT-4S proliferation), consisted of X-4G supernatant (containing IL-4) (100 μl), and 100 μl of a 5 x 10^4 cells/ml CT-4S cell suspension (5 x 10^3 cells/well).

Triplicate wells were made for each test, and a minimum of 6 wells was made for each control. Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere. Cells were pulsed with 20 μl of a 50 μCi/ml solution of methyl-tritiated thymidine, to make a final concentration of 1 μCi per well, and cultured for 18 hours at 37 °C in a humidified

5% CO_2 atmosphere. Incorporated label was harvested on glass fiber filters with a cell harvester, and the radioactivity was counted using a liquid scintillation spectrophotometer.

Calculations and Statistical Analysis

For tick biology studies, mean tick weight, mean salivary gland weight, and mean protein content of one salivary gland pair (± SE) were calculated, on each day of feeding, for each species (*D. andersoni*, *A. americanum* and *R. sanguineus*). The average weight of one gland obtained from each group of ten ticks was determined. Triplicate averages were used to determine the mean (± SE) for each tick species. Slopes of curves of tick weight, salivary gland weight, and salivary gland protein content were determined, to compare rates of increase among the three tick species.

For TNF-α assays, percentage-specific lysis was calculated according to the formula: [1-(O.D. of sample well/ O.D. of control well) X 100]
One unit of TNF-α activity is defined as the amount required for 50% lysis of L929 monolayer (Urbaschek & Urbaschek, 1987).

Mean cytotoxicities ± SE were calculated for three replicates with doubling dilutions, ranging from undiluted to 1:8. The dilution of the LPS-stimulated macrophage supernatant control, that had a cytotoxicity of 50%-60% (1:4 dilution in this case), was used for calculations. Mean percent suppression ± SE of TNF-α production was determined. Equality of variance among groups was determined using the F-test. Significant differences of O.D. values between each day of feeding, and the positive

control was determined by the appropriate two-tailed student's *t* test depending on whether the variances were equal or not.

For lymphocyte blastogenesis assays, IL-1, IL-2 or IL-4 assays, mean counts per minute (CPM) ± SE for each set of three replicates were calculated. Percent suppression for each sample well was determined using the formula:

% Suppression = [(Mean of +ve control - Sample reading)/ Mean of +ve control] x100

Mean percent suppression ± SE for IL-1, IL-2, or IL-4 production were determined. Equality of variance between triplicate wells for a certain day of feeding, and positive controls consisting of six to twelve wells were determined using the F-test. Significant differences of CPM between each day of feeding, and the positive control were determined by the appropriate two-tailed student's *t* test, either for equal variances, or for unequal variances depending on the outcome of the *F*-test.

Principles and procedures of statistical analysis used in this study were according to Steel and Torrie, (1980), and calculations and statistical analysis were done using spreadsheets in 'Microsoft Excel'. The AVERAGE function was used to calculate the mean of a sample of values. To calculate the standard error, the STDEV/ SQRT(n) function was used, were n was the number of observations in the sample. The standard error represents the deviation of the obtained mean value from the population mean.

The SLOPE function was used to determine the differences in rates of increase of tick weight, salivary gland weight, and protein content of SGE among the three tick species. The slope is the vertical distance divided by the horizontal distance between any two points on the line, which is the rate of change along the regression line

The FTEST function was used to determine the equality of variance between each day of feeding, and the positive control. If the returned probability value (P) associated with the *F*-test was greater than 0.05, variance was considered equal (homoscedastic), if $P<0.05$, then variance was considered unequal (heteroscedastic).

The TTEST function was used to determine significant differences between each day of feeding, and the positive control. This test determines whether two samples are likely to have come from the same two underlying populations, that have the same mean. The appropriate two-tailed Student's *t*- Test was used depending on the outcome of the *F*-test (either equal or unequal variances). Levels of significance were determined by calculating the probability value (P) associated with a Student's *t*-Test. If the value of P returned was more than 0.05, the means were not considered significantly different. If P was (0.01 to 0.05), the means were considered significantly different at a level of $P<0.05$, and if P is less than 0.01, the means were considered significantly different at a level of $P<0.01$.

CHAPTER III

RESULTS

Changes in Tick Weights During Feeding

The mean weight of unmated female *D. andersoni* was ≈14 mg on day zero, and this species attained a mean weight of ≈231 mg on day seven of feeding (an increase of ≈16 fold). The mean weight of female *A. americanum* was ≈ 4 mg on day zero, and this species attained a mean weight of ≈15 mg on day seven of feeding (an increase of ≈4 fold). The mean weight of female *R. sanguineus* was ≈ 2 mg on day zero, and attained a mean weight of ≈ 53 mg on day seven of feeding (an increase of ≈27 fold).

The changes in mean weights of unmated *D. andersoni, A. americanum* and *R. sanguineus* female ticks during days zero (unfed) to day seven of feeding are shown in Figure 1 & Table A-1 in Appendix A. *Dermacentor andersoni* mean weights doubled by day three of feeding, and showed the greatest rate of mean weight gain of the three tick species throughout days five to seven of feeding. *Rhipicephalus sanguineus* mean weights did not double until day four of feeding, but ticks showed a rate of weight gain throughout days five to seven of feeding. *Amblyommma americanum* mean weights did not double until day four of feeding, and gradually increased in mean weight to around four-fold by day seven of feeding.

The slope of the curve for the increase in mean tick weight for unmated female *D. andersoni* was the highest (28.6), followed by *R. sanguineus* (6.2), and *A. americanum* (1.7), indicting that the rate of increase in mean tick weight during days zero to seven of

feeding of unmated female *D. andersoni* was the most rapid among the three tick species, followed by *R. sanguineus*, then *A. americanum*.

Changes in Salivary Gland Wet Weights During Feeding

The mean wet weight of one pair of *D. andersoni* salivary glands was ≈1.2 mg on day zero, and attained a mean weight of ≈ 6.0 mg on day seven of feeding (an increase of ≈5 fold). The mean salivary gland weight of *A. americanum* was ≈ 0.3 mg on day zero, and attained a mean weight of ≈1.2 mg on day seven of feeding (an increase of ≈4 fold). The mean salivary gland weight of *R. sanguineus* was ≈ 0.1 mg on day zero, and attained a mean weight of ≈1.2 mg on day seven of feeding (an increase of ≈12 fold).

The changes in mean wet weights of one pair of unmated female *D. andersoni, A. americanum* and *R. sanguineus* salivary glands during days zero to seven of feeding is shown in Figure 2 & Table A-2 in Appendix A. *Dermacentor andersoni* salivary glands initially had the highest mean wet weight of the three tick species, followed by *A. americanum* then *R. sanguineus*. *Dermacentor andersoni* salivary glands attained the highest mean wet weight of the three tick species on day seven of feeding, followed by *A. americanum* and *R. sanguineus* that both attained a similar mean wet weight on day seven of feeding.

The slope of the curve for the increase in wet salivary gland weight for unmated female *D. andersoni* was the highest (0.7), followed by *A. americanum* (0.1), and *R. sanguineus* (0.1), indicating that the most rapid rate of increase in wet salivary gland

weight during days zero to seven of feeding was for unmated female *D. andersoni*, followed by *A. americanum* and *R. sanguineus*, which had similar rates of increase.

Changes in Protein Content of Salivary Gland Extracts During Feeding

The mean protein content of salivary gland extract (SGE) of one pair of *D. andersoni* salivary glands was ≈ 6.1 µg on day zero, and attained a mean of ≈138.8 µg on day seven of feeding (an increase of ≈23 fold). The mean protein content for *A. americanum* SGE was ≈ 4.0 µg on day zero, and attained a mean of ≈39.4 µg on day seven of feeding (an increase of ≈10 fold). The mean protein content for *R. sanguineus* SGE was ≈ 0.5 µg on day zero, and attained a mean of ≈ 45.5 µg on day seven of feeding (an increase of ≈86 fold).

The changes in mean protein content of SGE of one pair of salivary glands for unmated female *D. andersoni, A. americanum* and *R. sanguineus* ticks during days zero to seven of feeding are shown in Figure 3 & Table A-3 in Appendix A. *Dermacentor andersoni* SGE initially had the highest protein content of the three tick species, followed by *A. americanum* then *R. sanguineus.*

The slope of the curve for the increase in mean SGE protein content for unmated female *D. andersoni* was the highest (17.5), followed by *A. americanum* (5.7), and *R. sanguineus* (5.5), indicating that the rate of increase in mean SGE protein content for unmated female *D. andersoni* was the most rapid, followed by *A. americanum*, then *R. sanguineus.*

Feeding patterns for unmated females of the three tick species were different. The growth of *D. andersoni* mean tick weights, salivary gland weights and SGE protein contents were more rapid than either *A. americanum* or *R. sanguineus*, as is indicated by the higher slopes for *D. andersoni*. *A. americanum* and *R. sanguineus* are nearly equal in rates of growth of salivary gland weights, and salivary gland protein contents, although *R. sanguineus* had a higher rate of increase in tick weight than *A. americanum*, as indicated by the higher slope value.

Effect of SGE on Lymphocyte Blastogenesis in Response to Con A Stimulation

Dermacentor andersoni SGE (20μg protein/ml = 4μg SGE protein /5 x10^5 splenocytes /200μl medium /well) suppression of lymphocyte blastogenic responses of normal murine splenocytes to the T-lymphocyte mitogen Con A was ≈ 29 % for SGE obtained on day zero, was a maximum of ≈ 59% on day one of feeding, dropped to ≈ 39% on day three of feeding, and again rose to ≈ 50% on day four of feeding, and then dropped to ≈ 27% on day seven of feeding. Percent suppression was significant ($P<0.01$) on days zero to seven of feeding. *Amblyomma americanum* SGE suppression was ≈ 25% on day zero of feeding, dropped to ≈ 11% on day two, was a maximum of ≈ 71% on day five, and suppression was ≈ 66% on day seven of feeding. Percent suppression was significant ($P<0.01$) on days zero to one and three to seven of feeding. *Rhipicephalus sanguineus* SGE was not significantly suppressive on days zero to four of feeding, suppression was ≈ 24% on day five of feeding, and attained a maximum of ≈ 62% on day seven of feeding. Percent suppression was significant ($P<0.01$) on days five to seven of feeding.

Suppression of lymphocyte blastogenesis in response to Con A stimulation when incubated with 4 µg/well (20 µg/ml) of SGE varied among unmated females of the three tick species (Figure 4). Mean counts per minute are shown for the effect of *D. andersoni*, *A. americanum*, and *R. sanguineus* SGE on lymphocyte blastogenesis in response to Con A stimulation at concentrations of (20 µg/ml, 10 µg/ml & 5 µg/ml SGE protein) for days zero to seven of feeding in Appendix B: (Tables B-1, B-3 and B-5) respectively. Tables B-2, B-4 and B-6 show the mean percent suppression of proliferative responses of lymphocytes in response to Con A for *D. andersoni*, *A. americanum*, and *R. sanguineus* SGE respectively. A dilution effect on percent suppression can be observed for decreasing concentrations of *D. andersoni*, and *A. americanum* SGE on days zero to seven of feeding, while a dilution effect was observed for *R. sanguineus* SGE on days three to seven of feeding.

Effect of SGE on Lymphocyte Blastogenesis in Response to LPS Stimulation

Dermacentor andersoni SGE (20µg protein/ml = 4µg SGE protein /5 x10^5 splenocytes /200µl medium /well) suppression of lymphocyte blastogenic responses of normal murine splenocytes to the B-lymphocyte mitogen LPS was a maximum of ≈ 64% for SGE obtained on day zero and dropped to ≈ 11% on day seven of feeding. Percent suppression was significant ($P<0.01$) on days zero to six of feeding and significant ($P<0.05$) on day seven of feeding. *Amblyomma americanum* SGE suppression was ≈ 63% on day zero, was a maximum of ≈ 75% on day one, and decreased to ≈ 23% on day seven of feeding. Percent suppression was significant ($P<0.01$) on days zero to seven of

feeding. *Rhipicephalus sanguineus* SGE suppression was ≈ 9% day zero, was a maximum of ≈ 22% on day one, and was not suppressive on day seven of feeding. Percent suppression was significant ($P<0.01$) on days one to three, and significant ($P<0.05$) on day six of feeding.

Salivary gland extract of unmated females of all three tick species suppressed lymphocyte blastogenesis in response to LPS stimulation when incubated with 4 μg/well (20 μg/ml) of SGE (Figure 5). Mean counts per minute are shown for the effect of *D. andersoni*, *A. americanum*, and *R. sanguineus* SGE on lymphocyte blastogenesis in response to LPS at concentrations of (20 μg/ml, 10 μg/ml & 5 μg/ml protein) for days zero to seven of feeding in Appendix C: (Tables C-1, C-3 and C-5) respectively. Tables C-2, C-4 and C-6 show the mean percent suppression of proliferative responses of lymphocytes in response to LPS for *D. andersoni*, *A. americanum*, and *R. sanguineus* SGE respectively. A dilution effect on percent suppression can be observed for decreasing concentrations of *D. andersoni*, and *A. americanum* SGE on days zero to seven of feeding, while a dilution effect was observed for *R. sanguineus* SGE on days three and six of feeding.

Effect of SGE on Tumor Necrosis Factor-α (TNF-α) Production

The dilution of the LPS-stimulated macrophage supernatant (positive) control with a cytotoxicity of ≈50%-60% (1:4 dilution in this case), was used to calculate the results for TNF-α assays. Suppression of TNF-α production by normal murine peritoneal macrophages due to incubation with *D. andersoni* SGE (20 μg/ml = 20μg SGE protein /5

x10^5 macrophages /1 ml medium /well) was a maximum of ≈88% for SGE obtained on day zero, and went down to ≈51% on day two of feeding, and rose to ≈71% on day three of feeding, and was ≈ 40% on day seven of feeding. Percent suppression was significant ($P<0.01$) on days zero to seven of feeding. Suppression of TNF-α production by *A. americanum* SGE was ≈16 % on day zero of feeding, attained a maximum of ≈ 44% on day five of feeding, and went down to ≈ 6% on day seven of feeding. Percent suppression was significant ($P<0.01$) on days zero & two to six of feeding. Suppression of TNF-α production by *R. sanguineus* SGE was ≈10 % on day zero of feeding, attained a maximum of ≈17% on day two of feeding, and decreased to ≈ 9% on day seven of feeding. Percent suppression was significant ($P<0.05$) on days zero, two and three of feeding and significant ($P<0.01$) on day five of feeding.

Suppression of TNF-α production in response to LPS stimulation of macrophages when incubated with 20 µg/ml of SGE varied among unmated females of the three tick species (Figure 6). Mean counts per minute are shown for the effect of *D. andersoni*, *A. americanum*, and *R. sanguineus* SGE on TNF-α production at concentrations of (20 µg/ml, 10 µg/ml & 5 µg/ml protein) for days zero to seven of feeding in Appendix D: (Tables D-1, D-3 and D-5) respectively. Tables D-2, D-4, and D-6 show the mean percent suppression of TNF-α production for *D. andersoni*, *A. americanum*, and *R. sanguineus* SGE respectively. A dilution effect on percent suppression can be observed for decreasing concentrations of *D. andersoni* SGE on days zero to three of feeding, and *A. americanum* SGE on days zero, five and six of feeding, while a dilution effect was not observed for *R. sanguineus* SGE.

Effect of SGE on Interleukin-1 (IL-1) Production

Suppression of IL-1 production by normal murine peritoneal macrophages due to incubation with *D. andersoni* SGE (20 μg/ml = 20μg SGE protein /5 x10^5 macrophages /1 ml medium /well) was ≈ 76% for SGE obtained on day zero and attained a maximum of ≈ 97% on day two of feeding and was ≈ 87% on day seven of feeding. Percent suppression was significant ($P<0.01$) on days zero to seven of feeding. Suppression of IL-1 production by *A. americanum* SGE was a maximum of ≈ 84% on day zero of feeding, decreased to ≈ 48% on day four of feeding, and was ≈ 74% on day seven of feeding. Percent suppression was significant ($P<0.01$) on days zero to seven of feeding. Suppression of IL-1 production by *R. sanguineus* SGE was ≈ 38% on day zero of feeding, went down to ≈ 20% on day three, and attained a maximum of ≈ 61% on day five of feeding. *Rhipicephalus sanguineus* SGE suppression of IL-1 production was significant ($P<0.01$) on days zero to five of feeding. Production of IL-1 was enhanced significantly ($P<0.01$) on day six, and enhanced significantly ($P<0.05$) on day seven of feeding.

Suppression of *in vitro* IL-1 production in response to LPS stimulation of normal murine peritoneal macrophages when incubated with 20 μg/ml of SGE varied among unmated females of the three tick species (Figure 7). Mean counts per minute are shown for the effect of *D. andersoni*, *A. americanum*, and *R. sanguineus* SGE on IL-1 production at concentrations of (20 μg/ml, 10 μg/ml & 5 μg/ml protein) for days zero to seven of feeding in Appendix E: (Tables E-1, E-3 and E-5) respectively. Tables E-2, E-4, and E-6 show the mean percent suppression of IL-1 production for *D. andersoni*, *A.*

americanum, and *R. sanguineus* SGE respectively. A dilution effect on percent suppression can be observed for decreasing concentrations of *D. andersoni*, and *A. americanum* SGE on days zero to seven of feeding, while a dilution effect was observed for *R. sanguineus* SGE on days zero to five of feeding.

Effect of SGE on Interleukin-2 (IL-2) Production

Dermacentor andersoni SGE (20 μg/ml = 20 μg SGE protein /2.5 x10^6 splenocytes /1 ml medium /well) suppression of the *in vitro* production of the T_H1 cytokine IL-2 in response to Con A by normal murine splenocytes was a maximum of ≈19% for SGE obtained on day zero, ranged between ≈ 11% and ≈ 17% on days one to six of feeding, and went down to ≈ 6% on day seven of feeding. Percent suppression was significant ($P<0.01$) on days zero to two and four to six, and significant ($P<0.05$) on day three of feeding. *Amblyomma americanum* SGE suppression was ≈16% on day zero of feeding, went down to ≈ 6% on day one, no suppression was observed on days two to six, suppression attained a maximum of ≈ 33% on day seven of feeding. Percent suppression was significant ($P<0.01$) on days zero & seven of feeding. A significant ($P<0.01$) enhancing effect was observed for *R. sanguineus* SGE obtained on day six of feeding. *Rhipicephalus sanguineus* SGE suppression was ≈ 3% on day zero of feeding, suppression ranged between ≈ 1% and ≈ 8% on days one to five of feeding, attained a maximum of ≈ 8% on day six, and was not suppressive on day seven of feeding. Percent suppression was significant ($P<0.05$) on day six of feeding.

Suppression of *in vitro* production of the T_H1 cytokine IL-2 in response to Con A stimulation of normal murine splenocytes when incubated with 20 μg/ml of SGE varied among unmated females of the three tick species (Figure 8). Mean counts per minute are shown for the effect of *D. andersoni*, *A. americanum*, and *R. sanguineus* SGE on IL-2 production at concentrations of (20 μg/ml, 10 μg/ml & 5 μg/ml protein) for days zero to seven of feeding in Appendix F: (Tables F-1, F-3 and F-5) respectively. Tables F-2, F-4, and F-6 show the mean percent suppression of IL-2 production for *D. andersoni*, *A. americanum*, and *R. sanguineus* SGE respectively. A dilution effect on percent suppression can be observed for decreasing concentrations of *D. andersoni* SGE on days one, two, four and six of feeding, and *A. americanum* SGE on days zero-one and seven of feeding, while a dilution effect was observed for *R. sanguineus* SGE on days six of feeding.

Effect of SGE on Interleukin-4 (IL-4) Production

Dermacentor andersoni SGE (20 μg/ml = 20 μg SGE protein /2.5 $x10^6$ splenocytes /1 ml medium /well) suppression of *in vitro* production of the T_H2 cytokine IL-4 by normal murine splenocytes was ≈ 54% for SGE obtained on day zero, and attained a maximum of ≈ 71% on day one of feeding, went down to ≈ 49% on day one of feeding, rose to ≈ 63% on day five, and was ≈ 51% on day seven of feeding. Percent suppression was significant ($P<0.01$) on days zero to seven of feeding. *Amblyomma americanum* SGE suppression was ≈ 42% on day zero of feeding, went down to ≈ 5% on day two of feeding, attained a maximum of ≈ 94% on day seven of feeding. Percent

suppression was significant ($P<0.01$) on days zero to one and three to seven of feeding. *Rhipicephalus sanguineus* SGE suppression was not suppressive on day zero of feeding, was ≈12% on day one of feeding, attained a maximum of ≈ 74% on day seven of feeding. Percent suppression was significant ($P<0.01$) on days one & three to seven of feeding.

Suppression of *in vitro* production of the T_H2 cytokine IL-4 in response to Con A stimulation of normal murine splenocytes when incubated with 20 μg/ml of SGE varied among unmated females of the three tick species (Figure 9). Mean counts per minute are shown for the effect of *D. andersoni*, *A. americanum*, and *R. sanguineus* SGE on IL-4 production at concentrations of (20 μg/ml, 10 μg/ml & 5 μg/ml protein) for days zero to seven of feeding in Appendix G: (Tables G-1, G-3 and G-5) respectively. Tables G-2, G-4, and G-6 show the mean percent suppression of IL-4 production for *D. andersoni*, *A. americanum*, and *R. sanguineus* SGE respectively. A dilution effect on percent suppression can be observed for decreasing concentrations of *D. andersoni*, and *A. americanum* SGE on days zero to seven of feeding, while a dilution effect was observed for *R. sanguineus* SGE on days one and three to seven of feeding.

Summary of the Immunosuppressive Effects of SGE of *D. andersoni*, *A. americanum* and *R. sanguineus* on Lymphocyte Blastogenesis and Cytokines

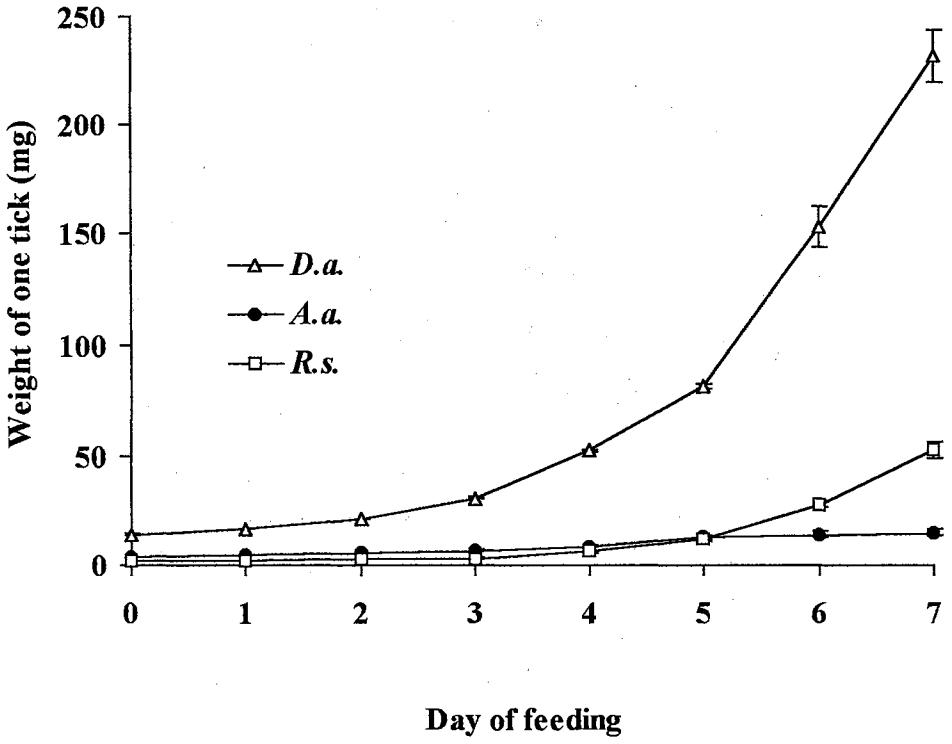
Results for lymphocyte blastogenesis and cytokine assays represent one time runs. All assays were repeated once, and although the second set of results gave similar overall patterns, they were not shown due to various problems in the protocols, controls, and materials used.

Immunosuppression of lymphocyte blastogenesis by normal murine splenocytes response to the T-lymphocyte mitogen Con A was initially higher for *D. andersoni* SGE, and increased with SGE obtained from later days of feeding, for *A. americanum* and *R. sanguineus*. Suppression of lymphocyte blastogenesis of normal murine splenocytes in response to the B-lymphocyte mitogen LPS was also observed, but this decreased with SGE obtained from later days of feeding for all three tick species.

Suppression of the elaboration of the cytokine TNF-α by normal murine peritoneal macrophages was initially highest for *D. andersoni* and gradually decreased with SGE obtained from later days of feeding, while suppression increased up to day 5 for *A. americanum* SGE, then decreased onwards, and suppression was relatively low for *R. sanguineus* SGE throughout feeding. Suppression of the elaboration of the cytokine IL-1 by normal murine peritoneal macrophages was higher for *D. andersoni* SGE up to day seven, followed by *A. americanum* SGE which also maintained its levels up to day seven, while suppression for *R. sanguineus* SGE were lower, and dropped off drastically after day five, where an enhancing effect on IL-1 on days six and seven was observed.

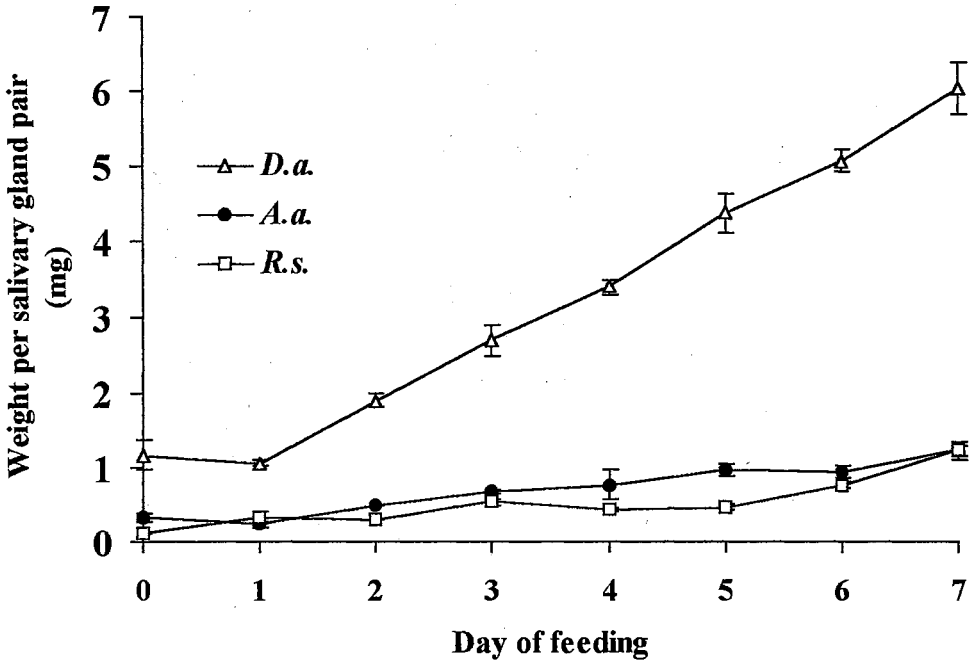
Suppression of the elaboration of T_H1 cytokine IL-2 from normal murine splenocytes, although significant, was low in magnitude for all three tick species. Significant suppression of the elaboration of the T_H2 cytokine IL-4 from normal murine splenocytes was observed, which was initially higher for *D. andersoni* SGE, and increased with SGE obtained from later days of feeding, for *A. americanum* and *R. sanguineus*.

Figure 1. Mean weight ± SE of unmated female ticks collected daily during feeding



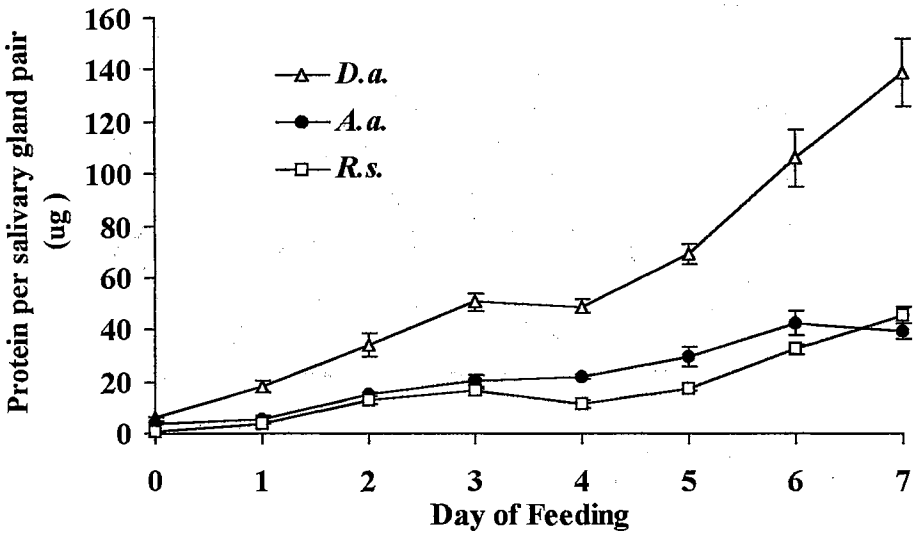
On each day of feeding, for each species, three groups of ten ticks each were weighed, and an average tick weight was obtained from each group. Each point represents the common mean tick weight and standard error values obtained from the tick weight averages of the three groups.

Figure 2. Mean wet weight ± SE of one pair of salivary glands from unmated female ticks



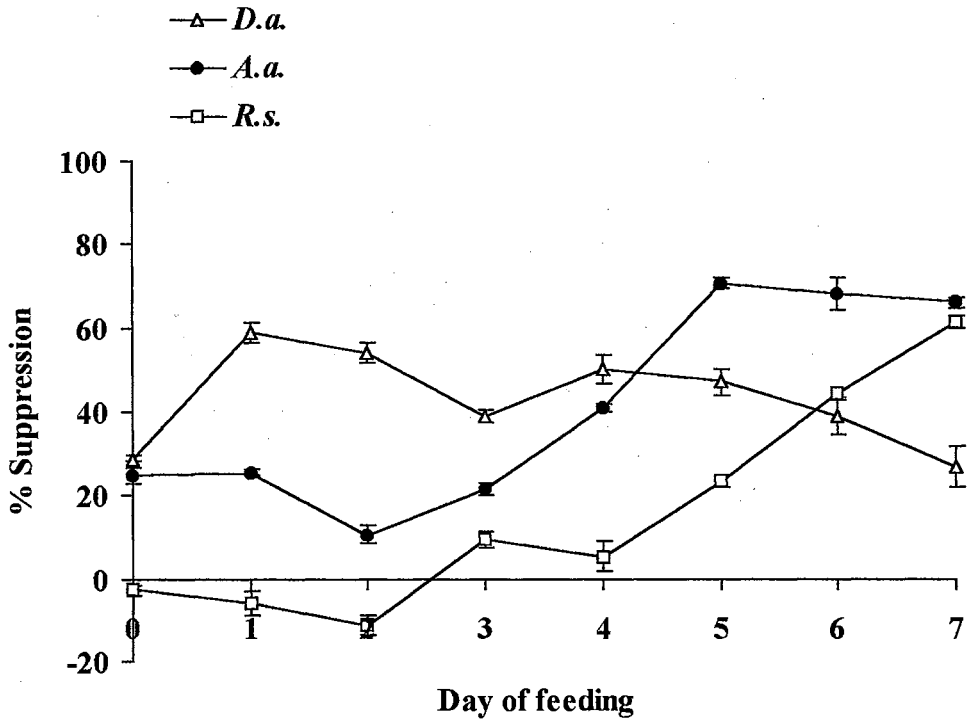
On each day of feeding, for each species, three groups of salivary gland pairs dissected from ten ticks each were weighed, and an average weight was obtained from each group. Each point represents the common mean salivary gland pair wet weight and standard error values obtained from the tick salivary gland wet weight averages of the three groups.

Figure 3. Mean protein content of salivary gland extract ± SE for one pair of salivary glands from unmated female ticks



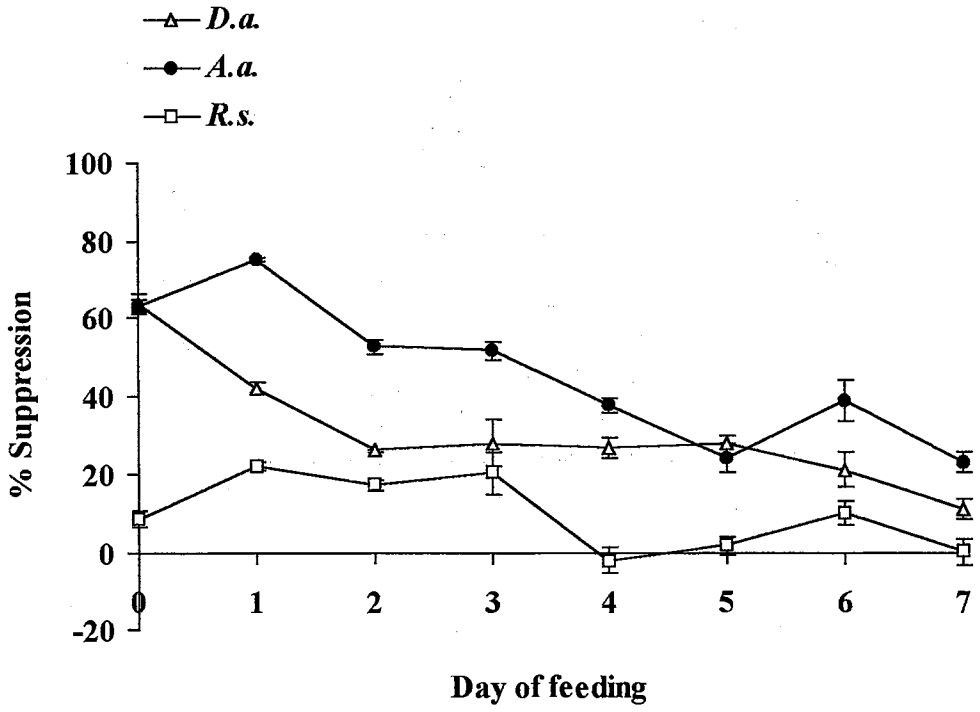
On each day of feeding, for each species, protein contents of three groups of SGE obtained from ten pairs of tick salivary glands were determined, and an average protein content for SGE of one salivary gland pair was obtained from each group. Each point represents the common mean protein content and standard error values obtained from the tick SGE protein content averages of the three groups.

Figure 4. Effect of salivary gland extracts, collected daily during feeding, on *in vitro* proliferative responses of normal murine splenocytes to the T-cell mitogen concanavalin A



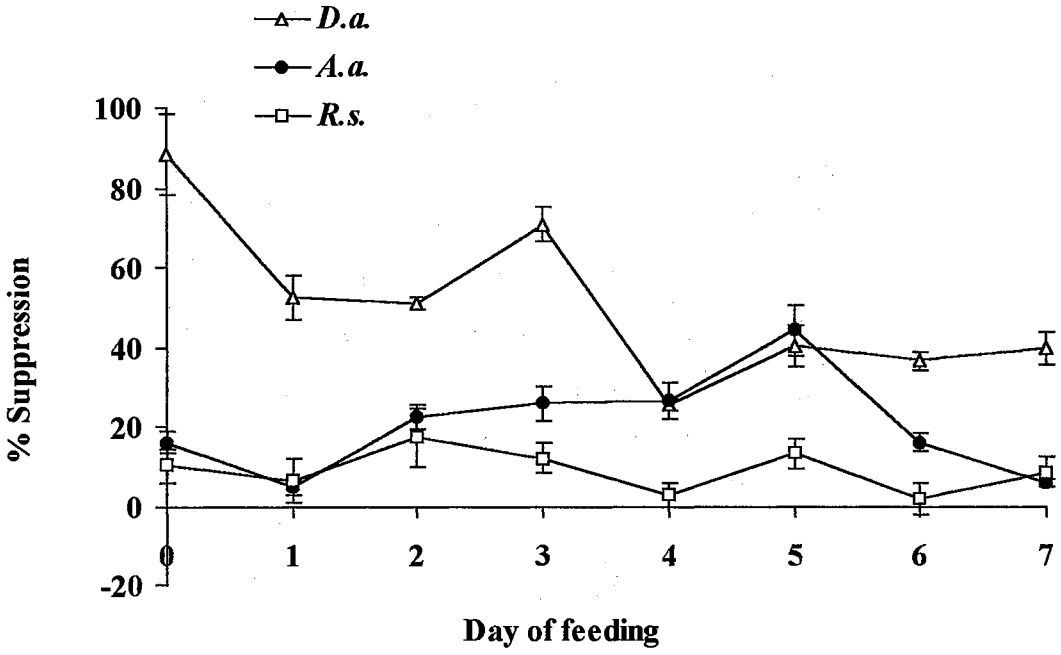
Percent suppression of *in vitro* lymphocyte blastogenesis in the presence of salivary gland extract protein (20μg/ml = 4μg SGE protein /5 x10^5 splenocytes /200μl medium /well) was calculated on the basis of mean CPM measured by blastogenesis of splenocytes treated with Con A only. Mean CPM were 283 ± 11 for unstimulated splenocytes and 145,956 ± 4,489 for cells stimulated with Con A. Negative values of "suppression" on the y-axis indicating an enhancement of lymphocyte blastogenesis were not significant at any point.

Figure 5. Effect of salivary gland extracts, collected daily during feeding, on *in vitro* proliferative response of normal murine splenocytes to the B-cell mitogen *Escherichia coli* lipopolysaccharide



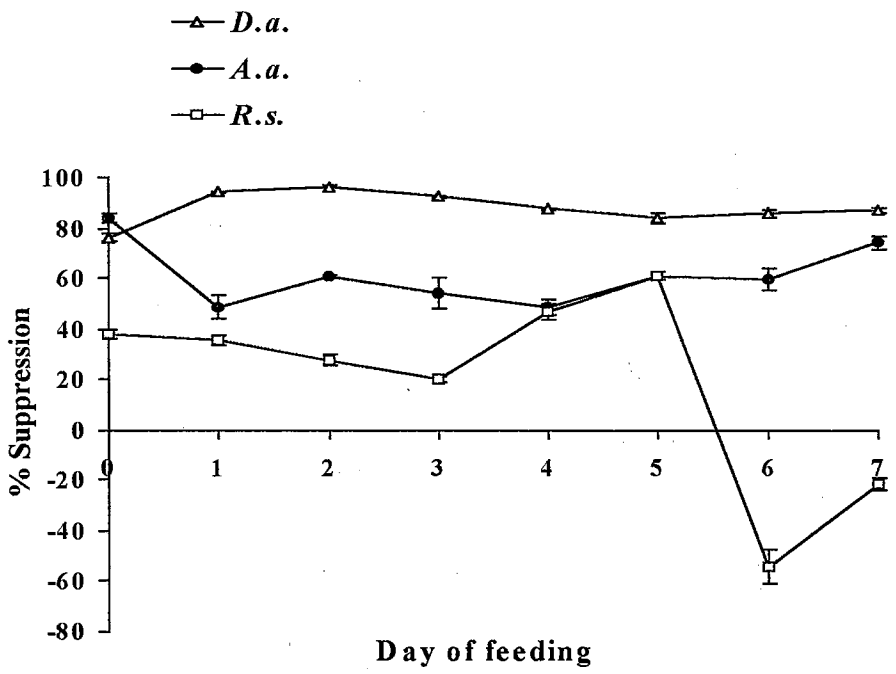
Percent suppression of *in vitro* lymphocyte blastogenesis in the presence of salivary gland extract protein (20μg/ml = 4μg SGE protein /5 x10^5 splenocytes /200μl medium /well) was calculated on the basis of mean CPM measured by blastogenesis of splenocytes treated with LPS only. Mean CPM were 283 ± 11 for unstimulated splenocytes and 36,678 ± 720 for LPS-stimulated cells. Negative values of "suppression" on the y-axis indicating an enhancement of lymphocyte blastogenesis were not significant at any point.

Figure 6. Effect of salivary gland extracts, collected daily during feeding, on *Escherichia coli* lipopolysaccharide-induced *in vitro* production of tumor necrosis factor-α by normal murine macrophages



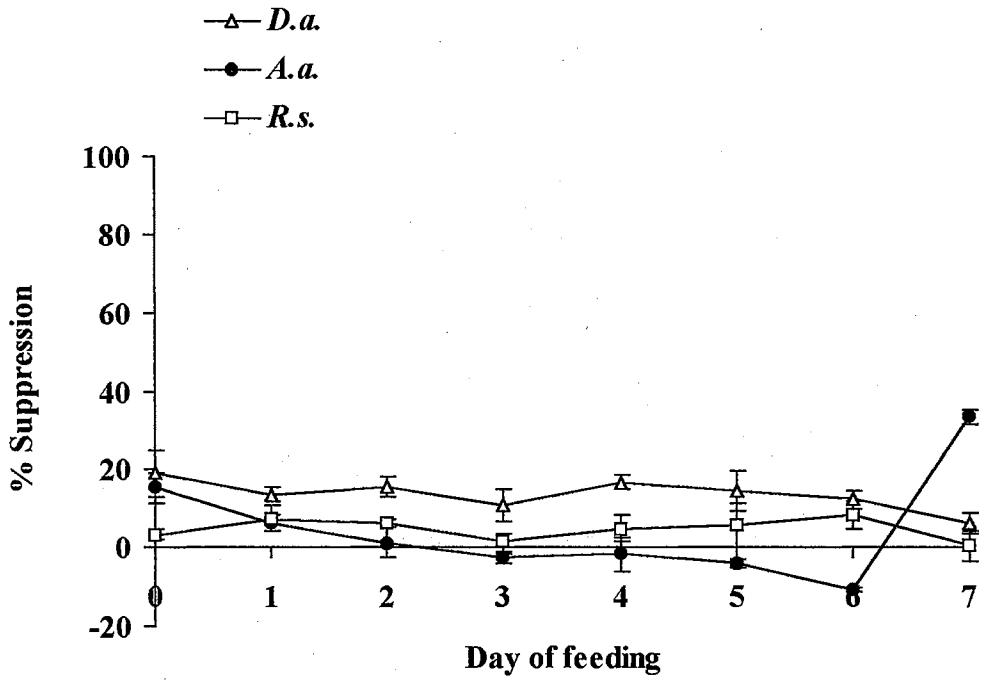
Percent suppression of TNF-α production in the presence of salivary gland extract protein (20 μg/ml = 20μg SGE protein /5 x10^5 macrophages /1 ml medium /well) was calculated on the basis of cytotoxicity to L929 cells measured in macrophage monolayer treated with LPS in the presence of actinomycin D. Mean cytotoxicity of the LPS-stimulated macrophage supernatants to L929 cells was 59.2% ± 1.3%. Unstimulated macrophage supernatants did not show any cytotoxicity to L929 cells.

Figure 7. Effect of salivary gland extracts, collected daily during feeding, on *Escherichia coli* lipopolysaccharide-induced *in vitro* production of interleukin-1 by normal murine macrophages



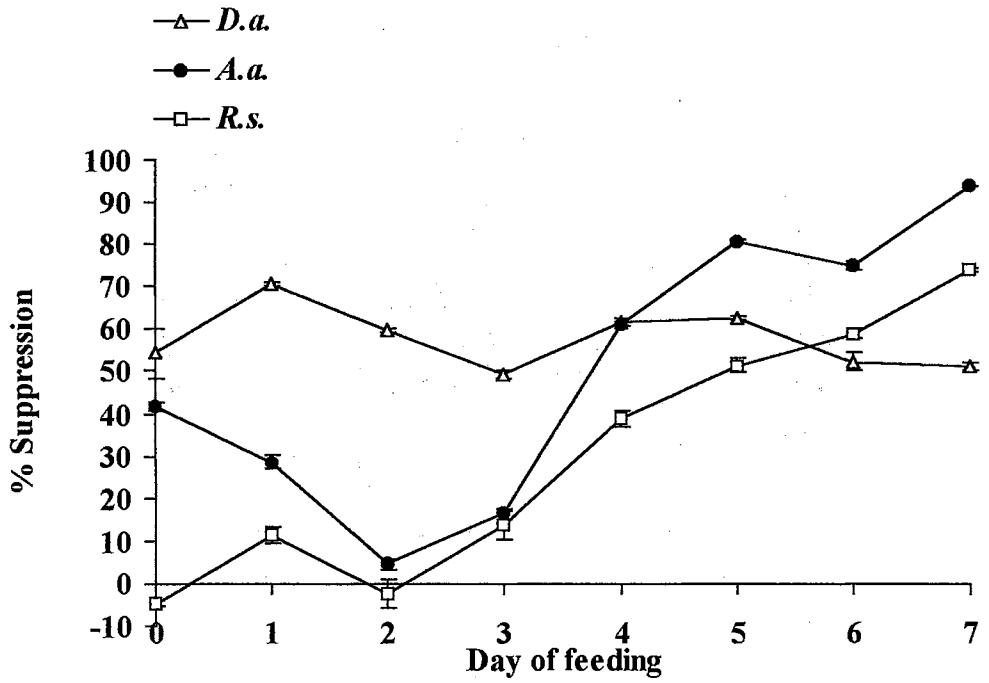
Percent suppression of IL-1 production in the presence of salivary gland extract protein (20 μg/ml = 20μg SGE protein /5 x10^5 macrophages /1 ml medium /well) was calculated on the basis of mean CPM measured by co-mitogenisis of C3H/HeJ mouse thymocytes incubated with Con A and LPS-stimulated macrophage supernatants (IL-1). Mean CPM ± SE were 117 ± 6 for unstimulated thymocytes, 3,579 ± 114 for thymocytes stimulated with Con A, 1,433 ± 61 for unstimulated macrophages and 6,734 ± 307 for macrophages stimulated with LPS. Significant negative values ($P<0.01$) of "suppression" indicated an enhancement of IL-1 elaboration due to incubation with *R. sanguineus* SGE obtained from ticks on days six and seven of feeding.

Figure 8. Effect of salivary gland extracts collected daily during feeding on concanavalin A-induced *in vitro* production of interleukin-2 by normal murine splenocytes



Percent suppression of IL-2 production in the presence of salivary gland extract protein (20 μg/ml = 20 μg SGE protein /2.5 x10^6 splenocytes /1 ml medium /well) was calculated on the basis of mean CPM measured by proliferation of CTLL-2 cells treated with Con A only. Mean CPM ± SE were 121 ± 8 for unstimulated CTLL-2 cells, and 214 ± 10 for unstimulated splenocytes, and 6,010 ± 92 for splenocytes stimulated with Con A. A significant ($P<0.01$) negative value of "suppression" on the y-axis indicated an enhancement of IL-2 elaboration due to incubation with *A. americanum* SGE obtained from ticks on days six of feeding.

Figure 9. Effect of salivary gland extracts collected daily during feeding on concanavalin A-induced *in vitro* production of interleukin-4 by normal murine splenocytes



Percent suppression of IL-4 production in the presence of salivary gland extract protein (20 μg/ml = 20 μg SGE protein /2.5 x10^6 splenocytes /1 ml medium /well) was calculated on the basis of mean CPM measured by proliferation of CT-4S cells treated with Con A only. Mean CPM ± SE were 409 ± 19 for unstimulated CT-4S cells, and 388 ± 15 for unstimulated splenocytes, and 23,238 ± 355 for splenocytes stimulated with Con A. Negative values of "suppression" on the y-axis indicating an enhancement of IL-4 production were not significant at any point.

CHAPTER IV

DISCUSSION

Immunomodulation of host responses is a common feature among several endoparasites including protozoa, (Liew, 1989) and helminths such as schistosomes (Stadecker, 1992). Immunosuppressive ability has also been observed in ectoparasitic hematophagous arthropods, either due to tick infestation (Wikel, 1982c ;Wikel & Osburn, 1982; Wikel, 1985; Inokuma *et al.*, 1993; Schorderet & Brossard, 1993; Urioste *et al.*, 1994), or by studying the effects on naive immunocompetent cells *in vitro* (Ramachandra & Wikel, 1992; Ramachandra & Wikel, 1995; Inokuma *et al.*, 1993).

Immunosuppression is not only a characteristic of long-term hematophagous arthropods such as ticks, but the salivary products of more transient blood-feeders, such as certain diptera may also modulate host immunological responses (Titus & Ribeiro, 1990; Cross *et al.*, 1993; Cross *et al.*, 1994a, 1994b). Immunosuppression by ticks is characterized by suppressed lymphocyte blastogenesis, reduced cytokine production by various cells during an immune responses, such as macrophages and T-lymphocytes (Ramachandra & Wikel, 1992; Ramachandra & Wikel, 1995), and reduced antibody responses to foreign antigens (Fivaz, 1989; Inokuma *et al.*, 1993; Wikel, 1985).

The molecules responsible for the different immunomodulatory effects of various tick species are not yet known, although a protein fraction (36-40 kDa) has been identified for *D. andersoni* ticks, that suppresses lymphocyte blastogenesis in response to the T-lymphocyte mitogen Con A (Bergman *et al.*, 1995). This discovery was the first isolation

and identification of tick salivary immunosuppressive activity by a protein fraction, and further studies will likely lead to similar discoveries for other tick species.

In this study, an *in vitro* model was used to help better understand differences in suppression of selected aspects of immune responses induced by ticks. Naive peritoneal macrophages and splenocytes were isolated from murine hosts, and incubated with salivary gland extracts, to see the effect on normal immune responses to stimulation *in vitro*. For example, LPS was used to activate macrophages, which simulates the activation *in vivo* when a macrophage encounters bacterial cells, although *in vitro* activation of macrophages by LPS is in a nonspecific manner (Wright, 1991). Mitogen-induced proliferation of T-lymphocytes can substitute for activation by immunogen-MHC complex formation (Sharon, 1983). Concanavalin A binds the T-lymphocyte receptor for antigen, and simulates *in vitro*, the activation *in vivo* of T-helper lymphocytes after they receive appropriate signals from activated macrophages (Gery *et al.*, 1972). Lipopolysaccharide-stimulation of splenocytes *in vitro* causes the activation and proliferation of B-lymphocytes, and their production of immunoglobulins (Dziarski, 1989).

Biological cytokine assays were chosen for this study over the commercially available enzyme-linked immunosorbant assays (ELISA), because ELISA will detect the specific epitopes on a molecule (Voller & deSavigny, 1981), whether the molecule is functional or not, while biological assays will only detect biologically active molecules thus, they are more indicative of the suppression of the actual function of the assayed cytokines. Also, transcription of cytokine mRNA can increase in the absence of protein release (Yau *et al.*, 1994), and bioassays detect the presence of biologically active

molecules because they are based on the biological functions of cytokines such as their ability to cause proliferation (Hamblin & O'Garra, 1987; Hu-Li *et al.*, 1989) or lysis (Issekutz & Bhimji, 1982; Koide & Steinman, 1987).

Salivary gland extracts were chosen for this study rather than saliva, because more protein can be obtained from glands, and salivary glands can be isolated more easily under sterile conditions than saliva. Protein content was used to quantitate the amount of SGE in this study because protein is thought to be responsible for immunosuppression of tick SGE. This idea is supported by the findings of several workers (Bergman *et al.*, 1995; Urioste *et al.*, 1994), and the fact that an immunosuppressive protein molecule has been purified from SGE of the tick *D. andersoni* (Bergman *et al.*, 1995).

Complex interactions among immunogens, antigen-presenting cells, lymphocytes, and soluble mediators such as cytokines and complement, are involved in the immune response against infectious agents and hematophagous arthropods (Willadsen, 1980; Wikel 1982a, 1988, 1996). The adaptive immune response can be divided into five stages: (1) uptake and processing of antigens, (2) transfer of information and activation of effector cells, (3) differentiation and proliferation of lymphocytes (4) synthesis and release of mediator substances (cytokines and antibodies); and (5) responses to mediators, antibodies and effector cells (Thomson *et al.*, 1992). Theoretically, it should be possible to suppress the immune response by intervening specifically at any of these stages.

Many immunosuppressive agents in current use, such as cyclophosphamide and methotrexate, are non-specific, cytotoxic compounds which interfere with DNA replication, and the production of lymphocytes, and other replicating cells (Thomson *et*

al., 1992). Some of these cytotoxic drugs are cell cycle phase specific such as azathioprine and methoterxate, which act during the S phase, or nonspecific such as cyclophosphamide, which affects both intermitotic and cycling cells, and these drugs are immunosuppressive when close to toxic levels. The mode of action of host immunosuppression by parasites, including ectoparasitic arthropods should not depend on cytotoxicity, because permanent damage to the host would not be to the benefit of a successful parasite.

The present study compares the effects of SGE of different tick species on lymphocyte blastogenesis, and cytokine elaboration by normal macrophages and lymphocytes. Such comparisons may help to understand differences in tick feeding patterns, and host ranges, and may even help to explain why different tick species vary in their ability to transmit various pathogens.

Some of the limitations of this study include the possibility of differences with results obtained if tick saliva was used instead of SGE. Tick saliva contains the actual substance secreted into the host during feeding. Tick saliva was not used in this study due to the difficulty of obtaining a sufficient amount of protein for the study, and also due to the possibility of contamination by substances such as dopamine, which are used for the induction of tick salivation. Even though the results obtained from *in vitro* studies on normal murine cell immune function may differ from the complex interactions that actually occur *in vivo*, these results can still provide a good model for the *in vivo* situation.

Percent suppression of lymphocyte blastogenesis in response to stimulation with the T-lymphocyte mitogen Con A was the highest for *D. andersoni* SGE during earlier days of feeding, than either *A. americanum* SGE or *R. sanguineus* SGE, but percent

suppression increased on later days for *A. americanum* SGE, and *R. sanguineus* SGE, indicating that T-helper lymphocyte functions are altered for all three tick species. The results obtained in this study are consistent with the findings of Ramachandra & Wikel, (1992), where *D. andersoni* SGE suppression of splenocyte blastogenesis in response to Con A was observed. T-lymphocytes are important for the regulatory, and effector pathways involved in both cellular, and humoral acquired immune responses (Kupfer & Singer, 1993), and the suppression of T-lymphocyte activation will inhibit their ability to carry out these functions.

Dermacentor andersoni and *A. americanum* SGE obtained from early days of feeding showed a higher percent suppression of *in vitro* blastogenesis of B-lymphocytes in response to LPS stimulation than *R. sanguineus* SGE. All three tick species showed a higher percent suppression of lymphocyte blastogenesis in response to the B-lymphocyte mitogen LPS for tick SGE, collected on earlier days, and the suppression rapidly declined during the course of feeding. Ramachandra & Wikel, (1992) reported that suppression of splenocyte proliferative responses to LPS was not observed, although their results actually indicated a suppressive response (negative percent change in counts per minute) for *D. andersoni* SGE (10 μg/ml) obtained from ticks on days zero to three of feeding, which is consistent with the results obtained in this study. The suppression of the activation of B-lymphocytes and antibody formation during the first days of tick feeding can suppress the rapid activation of memory B-lymphocytes that may be present due to a prior exposure to tick infestation, thus reducing immune responses that hinder tick attachment and commencement of feeding.

Percent suppression of TNF-α production was higher for *D. andersoni* than for either *A. americanum* or *R. sanguineus.* These findings are consistent with the studies done by Ramachandra & Wikel, (1992), on *D. andersoni* SGE which was found to be highly suppressive for TNF-α production. Tumor necrosis factor-α has several biological functions such as the induction and expression of class I and class II MHC molecules, activation of polymorphonuclear leukocytes (PMN), and anti-viral activity (Beutler & Cerami, 1989).

Dermacentor andersoni SGE showed a higher percent suppression of IL-1 production than *A. americanum* SGE, which in turn showed a higher suppression than *R. sanguineus* SGE. Similar results were obtained for *D. andersoni* SGE by Ramachandra & Wikel, (1992), which was found to be highly suppressive for IL-1 production. Interleukin-1 has several biological functions (Durum *et al.*, 1985). Interleukin-1 is important for the initiation of an acquired immune response, and for the mediation of inflammation (Mizel, 1987; Oppenheim & Gery, 1982). Interleukin-1 is a cofactor for both T-lymphocyte (Larsson *et al.*, 1980; Smith *et al.*, 1980), and B-lymphocyte activation (Hoffman, 1980; Howard & Paul, 1983), it induces lymphokine production by T-lymphocytes, promotes hematopoiesis (Bagby, 1989; Fibbe & Falkenburg, 1990; Stanley *et al.*, 1986; Van Damme & Billiau, 1987), and activates neutrophils and vascular endothelial cells (Klempner *et al.*, 1978; Luger *et al.*, 1983; Merriman *et al.*, 1977).

All three tick species had the ability to suppress both of the macrophage cytokines TNF-α and IL-1. Macrophages are one of the first cells that come into contact with foreign antigen, and these cells when activated, engulf, process and present antigen to

helper T-lymphocytes. Langerhans cells in the epidermis process immunogenic molecules such as proteins (Streilein *et al.*, 1990), and migrate to the draining lymph nodes to become dendritic cells (Steinman, 1993), and present these molecules to antigen-specific T-lymphocytes in the context of class II MHC molecules on the surface of the antigen-presenting cells (Liu & Janeway, 1991). Macrophages release the cytokines TNF-α and IL-1 as a result of this activation. The macrophage-derived cytokines, IL-1 and TNF-α are important regulators of the immune response (Dinarello *et al.*, 1986). Lowered levels of TNF-α and IL-1 can affect the immune response, and the inflammatory response to the tick, thus affecting the ability of the host to reject the feeding tick, and may also hinder the ability of the tick to transmit pathogens.

The suppression of IL-2 production by Con A-stimulated splenocytes was low for all three tick species, indicating that the direct effect on lymphocytes of the TH1 subset is minimal. These results resemble those obtained by Ramachandra & Wikel, (1992), where the suppression of IL-2 secretion by Con A-stimulated splenocytes was not as high as the suppression observed for macrophage-cytokines. In spite of this observation, the function of IL-2 could still be impaired indirectly due to suppression of macrophage cytokines such as IL-1 by tick SGE. Interleukin-1 provides necessary signals for the activation of T-lymphocytes, and the production of IL-2 by T_H1 lymphocytes (Larsson *et al.*, 1980; Smith *et al.*, 1980). Cell-mediated responses are regulated by T_H1 lymphocytes (Mosmann & Coffman, 1993), and the cutaneous basophil hypersensitivity reaction (CBH) commonly seen with tick infestation is a type of delayed hypersensitivity reaction that is mediated by T_H1 lymphocytes (Mosmann & Coffman, 1993). The suppression of IL-2 production will

hinder the autocrine stimulation of T-lymphocyte growth (which is an important function of IL-2). The clonal expansion of T-lymphocytes is important for the generation of cell-mediated immune responses, B-lymphocyte growth and clonal expansion, and the production of immunoglobulins, which will all be suppressed with reduced levels of IL-2 production.

Production of the T_H2 cytokine IL-4 was suppressed by *D. andersoni* SGE earlier, and for a longer time than for either *A. americanum* SGE or *R. sanguineus* SGE. Interestingly, the suppression of IL-4 activity by SGE of the three tick species (Figure 9) somewhat paralleled the suppression of lymphocyte blastogenesis in response to the T-lymphocyte mitogen Con A (Figure 4). This is the first report of the suppression of the T_H2 cytokine IL-4 by ticks. Wikel, (1985) observed a reduction in hemolytic plaque-forming cell immunoglobulin M (IgM) response to a thymic-dependent antigen for guinea pigs infested with *D. andersoni*. This observation supports the possibility of suppression of T_H2 responses by B-lymphocyte activators such as IL-4. The activation and differentiation of B-lymphocytes, and the production of circulating and homocytotropic antibodies specific for tick antigens is regulated by T_H2 lymphocytes (Mosmann & Coffman, 1993). Interleukin-4 causes activation, proliferation, and differentiation of antigen-specific B-lymphocytes (Stein *et al.*, 1986; Vitetta *et al.*, 1985), is a growth factor for T-lymphocytes and mast cells (Mosmann *et al.*, 1986; Schmitt *et al.*, 1986; Smith & Rennick, 1986), and exerts other effects on granulocytes, megakaryocytes, erythrocyte precursors, and macrophages (Crawford *et al.*, 1987; Meltzer *et al.*, 1987; Paul & Ohara, 1987; Peschell *et al.*, 1987; Zlotnick *et al.*, 1986). Interleukin-4 regulates B-lymphocyte

growth and expression of cell surface antigens such as Class II MHC which it up-regulates (Noelle *et al.*, 1984; Roehm *et al.*, 1984). Interleukin-4 also regulates the development of the homocytotropic antibodies, IgE and immunoglobulin G_1 (IgG_1) by B-lymphocytes (Snapper *et al.*, 1988a, b; Vitetta *et al.*, 1985). Mast cells and basophils become armed with homocytotropic antibodies that occupy Fc receptors on their surface (Beaven & Metzger, 1993), and these cells degranulate when salivary immunogens complex with the homocytotropic antibodies (Brossard & Girardin, 1979; Brossard *et al.*, 1982; Whelen & Wikel, 1993). Bioreactive molecules such as histamine, prostaglandins, and leukotrienes are released from the granules causing increased vascular permeability, and the accumulation of cells, complement and immunoglobulins at the bite site (Metzger & Kinet, 1988; Moore & Dannenberg, 1993). All the above functions may be impaired if IL-4 production is suppressed.

Salivary gland extracts from different tick species varied in their ability to suppress host cytokine elaboration, and lymphocyte blastogenesis. The ability to suppress cytokine production and lymphocyte blastogenesis seems to correlate with the ticks' feeding pattern. Rates of increase of mean tick weight, mean salivary gland weight and mean protein content of SGE were generally higher for unmated female *D. andersoni* than either unmated *A. americanum* or unmated *R. sanguineus* during days zero to seven of feeding on ovine hosts. *Dermacentor andersoni* females normally take up to 17 days to engorge, while *A. americanum* females may require up to 24 days, and *R. sanguineus* may require up to 50 days to engorge (United States Department of Agriculture, 1976). Salivary gland extracts of *D. andersoni* glands obtained on earlier days of feeding suppressed the

production of the macrophage cytokines TNF-α and IL-1 *in vitro* more than *A. americanum*, and the later tick species suppressed these cytokines at a higher level than *R. sanguineus*. Although IL-2 levels were not reduced as much as macrophage cytokines, the reduction of macrophage cytokine production, such as TNF-α and IL-1 is correlated with the modification of the T_H1 subset of helper T-lymphocytes (Abbas *et al.*, 1991), and should thus have an indirect effect on IL-2 production. Reduction of IL-4 production indicates that T_H2 subpopulation function is also affected. The suppression of lymphocyte blastogenesis in response to stimulation with the T-lymphocyte mitogen Con A indicates that the functions of T-helper lymphocytes are altered. Higher suppression of lymphocyte blastogenesis in response to the B-lymphocyte mitogen LPS by tick SGE on earlier days could indicate that B-lymphocyte responses are suppressed earlier on during feeding. Suppression seems to focus on T-lymphocyte responses later on during feeding, especially for *A. americanum* and *R. sanguineus*.

The immunosuppression observed in this study correlates with the established mechanisms of acquired resistance to ticks. Suppression of the production of the macrophage cytokines TNF-α and IL-1 will suppresses the functions of macrophages including: antigen presentation, and the regulation of B- and T-lymphocyte immune responses. The observed magnitude of suppression of the T_H1 cytokine IL-2 was to a lower extent than that of macrophage cytokines, but IL-2 production can be impaired indirectly due to suppression of macrophage cytokines such as IL-1 by tick SGE. The suppression of IL-2 production can inhibit expansion of antigen-specific T-lymphocyte clones, B-lymphocyte differentiation, and delayed hypersensitivity reactions. A

considerable suppression of the T_H2 cytokine IL-4 was observed, while previous studies did not reveal any alteration of IL-4 studies by tick salivary-gland extracts (Wikel, 1996). More studies need to be done to resolve this discrepancy. The suppression of IL-4 will lead to a suppression of antibody responses, which have been shown to be important factors for resistance to *R. appendiculatus* (Shapiro *et al.*, 1986), in guinea pigs. Serum γ globulin level of tick-infested bovines showed a negative correlation to mean engorgement weight of *Boophilus decoloratus*, supporting the importance of TH2 cytokines, B-lymphocyte function, and antibodies in acquired resistance to ticks (Rechav *et al.*, 1991).

Results from this study show that different tick species may have variable abilities to suppress the activation of both cell-mediated, and humoral immune responses of their hosts. From an evolutionary viewpoint, modulation represents an adaptation by the vector to circumvent the host immune defenses, that impair the tick's ability to successfully obtain a blood meal, such as immunological responses directed against the vector. Ticks, like many hematophagous arthropods also possess salivary components that can modulate other host defense mechanisms, including hemostasis and inflammation (Gordon & Allen, 1991; Limo *et al.*, 1991; Ribeiro *et al.*, 1985a; Ribeiro, 1987a; Ribeiro & Spielman, 1986). Different tick species vary in their host ranges, and feeding patterns, and this may be reflected in their ability to suppress immune responses. Both of the tick species *D. anderoni*, and *A. americanum* have a broad range of hosts in nature (Cupp, 1991), which includes ovine hosts. *Rhipicephalus sanguineus* ticks, are ectoparasites of canines (Cupp, 1991), and do not normally feed on sheep. The results obtained for *R. sanguineus* from feeding on an ovine host may differ from those that would be obtained when using canine

hosts. Differences may also be detected when comparing the effects of tick SGE on the cells of the natural hosts (for example, when using canine cells for *R. sanguineus*), rather than murine cells, but standardized assays must first be developed, if this is to be attempted.

Studies that should follow this study include the comparison of immunosuppressive effects of SGE of these tick species on other cytokines including the T_H2 IFN-γ. Interferon-γ is which is important for macrophage and NK cell activation (Trinchieri & Perussia, 1985), anti-viral activity and induction of the expression of class I and class II MHC molecules (Gerrard *et al.*, 1988), was suppressed by *D. andersoni* SGE (Ramachandra & Wikel, 1992). The development of new cytokine assay protocols will allow the comparison the ability of tick salivary gland extracts to suppress a wide range of cytokines. Such studies can help to explain differences in host ranges of different tick species, and differences their ability to transmit various pathogens. Studies of the effect of tick infestation on cytokine patterns at tick feeding sites, such as the *in vivo* studies done by Mbow (1994a, b, c) can also shed more light on the effect of tick salivary secretions on the local immune response. The introduction of various cytokines, either in the host circulation, or into the bite site, or the elimination of certain cytokines, can help to more fully understand the mechanisms of resistance to different tick species. Most of the studies on tick immunomodulation of host responses emphasized the relative importance of the suppression of T_H1 responses over T_H2 responses (Ramachandra & Wikel, 1992, Wikel, 1996), a suppression of T_H2 responses was also observed (Wikel, 1985). A more complicated case than the T_H1/ T_H2 switch observed with internal parasites such as

Leishmania may be the case (Heinzel *et al.*, 1989), and more studies must be done to clarify the situation with ticks. Although there have been several studies that have helped to better understand the interactions of ticks, their hosts, and the pathogens they transmit, more work has to be done before a clear picture of these complex interactions emerges.

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APPENDICES

APPENDIX A

Mean values ± SE for tick biology studies
(tick weight, salivary gland weight and protein content of salivary gland extracts) of
unmated female *D. andersoni*, *A. americanum* and *R. sanguineus*
collected daily during feeding

Table A-1. Mean tick weight ± SE (mg)

	Tick weight (mg)		
	Dermacentor andersoni	*Amblyomma americanum*	*Rhipicephalus sanguineus*
Day of feeding	MEAN ± SE	MEAN ± SE	MEAN± SE
0	14.13 ± 0.26	3.97 ± 0.15	1.97 ± 0.03
1	16.73 ± 0.26	4.83 ± 0.12	2.07 ± 0.03
2	21.23 ± 0.35	5.70 ± 0.06	2.37 ± 0.03
3	30.60 ± 0.38	6.71 ± 0.54	3.20 ± 0.15
4	52.53 ± 0.50	8.63 ± 0.44	6.27 ± 0.64
5	81.33 ± 1.19	12.72 ± 0.94	11.67 ±0.86
6	153.10 ± 9.20	13.98 ± 1.27	28.07 ± 1.14
7	231.20 ± 12.05	15.02 ± 1.31	52.83 ± 3.70

Mean tick weights are obtained by determining the common mean of 3 means for groups of 10 ticks each, and then determining the standard error (SE) of the common mean using the 3 mean values.

Table A-2. Mean salivary gland weight ± SE (mg)

Salivary gland weight (mg)			
	Dermacentor andersoni	*Amblyomma americanum*	*Rhipicephalus sanguineus*
Day of feeding	MEAN ± SE	MEAN ± SE	MEAN± SE
0	1.17 ± 0.20	0.33 ± 0.05	0.10 ± 0.00
1	1.07 ± 0.05	0.23 ± 0.05	0.33 ± 0.08
2	1.90 ± 0.10	0.50 ± 0.00	0.30 ± 0.00
3	2.70 ± 0.20	0.68 ± 0.04	0.53 ± 0.03
4	3.40 ± 0.10	0.77 ± 0.20	0.43 ± 0.03
5	4.37 ± 0.25	0.98 ± 0.08	0.47 ± 0.03
6	5.07 ± 0.15	0.94 ± 0.08	0.77 ± 0.03
7	6.03 ± 0.35	1.24 ± 0.07	1.23 ± 0.12

Mean salivary gland pair weights are obtained by determining the common mean of 3 means for groups of 10 ticks each, and then determining the standard error (SE) of the common mean using the 3 mean values.

Table A-3. Mean protein content (μg) of SGE from one salivary gland pair

Protein content of salivary gland extract (μg)			
	Dermacentor andersoni	*Amblyomma americanum*	*Rhipicephalus sanguineus*
Day of feeding	MEAN ± SE	MEAN ± SE	MEAN ± SE
0	6.11 ±0.21	3.98 ± 0.29	0.53 ± 0.03
1	18.39 ± 2.16	5.68 ± 0.87	3.88 ± 0.36
2	34.16 ± 4.47	15.08 ± 0.65	12.98 ± 1.51
3	50.60 ± 3.47	20.52 ± 1.97	16.82 ± 1.00
4	48.76 ± 2.68	21.95 ± 0.75	11.56 ± 1.55
5	69.01 ± 3.43	29.25 ± 3.75	17.25 ± 0.89
6	105.90 ± 11.20	42.55 ± 4.44	32.30 ± 1.86
7	138.80 ± 12.62	39.38 ± 3.19	45.49 ± 2.68

Mean SGE protein content for one tick salivary gland pair is obtained by determining the common mean of 3 means for groups of 10 ticks each, and then determining the standard error (SE) of the common mean using the 3 mean values.

APPENDIX B

Mean values ± SE for lymphocyte blastogenesis and cytokine assays with 3 concentrations of salivary gland extracts for *D. andersoni*, *A. americanum* and *R. sanguineus*
Lymphocyte Blastogenesis Assays-Concanavalin A

Table B-1. Effect of *Dermacentor andersoni* salivary gland extracts, collected daily during course of engorgement on proliferative response of normal murine splenocytes to the mitogen concanavalin A: Mean counts per minute (CPM ± SE) †

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	CPM ± SE	CPM ± SE	CPM ± SE
0	103,543 ± 3,440	125,022±15,270	126,895 ± 2,401
1	59,962 ± 414	128,389± 0,869	135,748 ± 4,624
2	67,098 ± 1,886	121,998 ± 3,143	126,703 ± 3,508
3	88,942 ± 261	119,286 ± 4,522	142,165 ± 2,400
4	72, 553 ± 3,118	106,887 ± 3,407	124,253 ± 2,514
5	77, 182 ± 1,693	106,948 ± 7,516	106,007±11,693
6	88,997 ± 2,165	120,004 ± 2,130	123,001 ± 5,187
7	106,942 ± 5,266	114,260 ± 4,381	125,707 ± 7,578

† Mean counts per minute (CPM± SE) for unstimulated splenocytes were 283 ± 11, whereas CPM± SE for splenocytes stimulated with con-A were 145,956 ± 4,489.

Table B-2. Effect of *Dermacentor andersoni* salivary gland extracts, collected daily during course of engorgement on proliferative response of normal murine splenocytes to the mitogen concanavalin A: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	** 29.1 ± 2.4	14.3 ± 10.5	13.1 ± 1.6
1	** 58.9 ± 0.3	12.0 ± 7.4	7.0 ± 3.2
2	** 54.0 ± 1.3	16.4 ± 2.2	13.2 ± 2.4
3	** 39.1 ± 0.2	18.3 ± 3.1	2.6 ± 1.6
4	** 50.3 ± 2.1	26.8 ± 2.3	14.9 ± 1.7
5	** 47.1 ± 1.2	26.7 ± 5.1	27.4 ± 8.0
6	** 39.0 ± 1.5	17.8 ± 1.5	15.7 ± 3.6
7	** 26.7 ± 3.6	21.7 ± 3.0	13.9 ± 5.2

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine splenocytes stimulated with Con A.

Table B-3. Effect of *Amblyomma americanum* salivary gland extracts, collected daily during course of engorgement on proliferative response of normal murine splenocytes to the mitogen concanavalin A: Mean counts per minute (CPM ± SE) †

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	CPM ± SE	CPM ± SE	CPM ± SE
0	109,376 ± 5,708	116,446 ± 1,619	121,195 ± 323
1	108,479 ± 977	124,485 ± 1,902	144,674 ± 5,138
2	130,322±10,345	152,312 ± 1,790	153,400 ± 1,455
3	114,339 ± 3,818	138,282 ± 5,793	142,402 ± 7,538
4	86,015 ± 1,114	125,338± 4,794	144,077 ± 928
5	43,003 ± 1,856	119,165 ± 2,869	131,902 ± 1,718
6	46,454 ± 2,992	111,243 ± 2,860	131,016 ± 3,156
7	49,688 ± 2,392	113,513 ± 4,223	129,300 ± 1,109

† Mean counts per minute (CPM± SE) for unstimulated splenocytes were 283 ± 11, whereas CPM± SE for splenocytes stimulated with con-A were 145,956 ± 4,489.

Table B-4. Effect of *Amblyomma americanum* salivary gland extracts, collected daily during course of engorgement on proliferative response of normal murine splenocytes to the mitogen concanavalin A: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	** 25.1 ± 3.9	20.2 ± 1.1	17.0 ± 0.2
1	** 25.7 ± 0.7	14.7 ± 1.3	0.9 ± 3.5
2	10.7 ± 7.1	-	-
3	** 21.7 ± 2.6	5.3 ± 4.0	2.4 ± 5.2
4	** 41.1 ± 0.8	14.1 ± 3.3	1.3 ± 0.6
5	** 70.5 ± 1.3	18.4 ± 2.0	9.6 ± 1.2
6	** 68.2 ± 2.0	23.8 ± 2.0	10.2 ± 2.2
7	** 66.0 ± 1.6	22.2 ± 2.9	11.4 ± 0.8

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine splenocytes stimulated with Con A.

Table B-5. Effect of *Rhipicephalus sanguineus* salivary gland extracts, collected daily during course of engorgement on proliferative response of normal murine splenocytes to the mitogen concanavalin A: Mean counts per minute (CPM ± SE) †

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	CPM ± SE	CPM ± SE	CPM ± SE
0	150,057±10,516	142,666 ± 5,236	136,536 ± 3,818
1	154,539 ± 9,184	157,012 ± 3,107	145,872 ± 1,725
2	162,439 ± 6,024	160,622 ± 5,908	153,935 ± 2,503
3	132,014 ± 7,886	149,928 ± 3,223	144,704 ± 1,946
4	138,114 ± 976	155,362 ± 4,071	146,246 ±2,689
5	111,364 ± 4,114	147,908 ± 1,617	145,032 ± 7,199
6	81,432 ± 6,111	127,038 ± 5,322	137,007 ± 4,485
7	56,171 ± 1,313	117,157 ± 2,255	122,460 ± 1651

† Mean counts per minute (CPM± SE) for unstimulated splenocytes were 283 ± 11, whereas CPM± SE for splenocytes stimulated with con-A were 145,956 ± 4,489.

Table B-6. Effect of *Rhipicephalus sanguineus* salivary gland extracts, collected daily during course of engorgement on proliferative response of normal murine splenocytes to the mitogen concanavalin A: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	-	2.3 ± 3.6	6.4 ± 2.6
1	-	-	0.1 ± 1.2
2	-	-	-
3	9.6 ± 5.4	-	0.9 ± 1.3
4	5.4 ± 0.7	-	-
5	** 23.7 ± 2.8	-	0.6 ± 4.9
6	** 44.2 ± 4.2	13.0 ± 3.6	6.1 ± 3.1
7	** 61.5 ± 0.9	19.7 ± 1.5	16.1 ± 1.1

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine splenocytes stimulated with Con A.

APPENDIX C

Mean values ± SE for lymphocyte blastogenesis and cytokine assays with 3 concentrations of salivary gland extracts for *D. andersoni*, *A. americanum* and *R. sanguineus*
Lymphocyte Blastogenesis Assays-Lipopolysaccharides

Table C-1. Effect of *Dermacentor andersoni* salivary gland extracts, collected daily during course of engorgement on proliferative response of normal murine splenocytes to *Escherichia coli* lipopolysaccharide: Mean counts per minute (CPM ± SE) ‡

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	CPM ± SE	CPM ± SE	CPM ± SE
0	13,276 ± 922	17,685 ± 87	22,007 ± 540
1	21,307 ± 478	28,384 ± 781	35,830 ± 1,051
2	27,089 ± 112	35,214 ± 2,944	39,429 ± 1,804
3	26,447 ± 2,182	36,950 ± 2,479	38,441 ± 833
4	26,869 ± 868	37,569 ± 888	39,214 ± 1,449
5	26,538 ± 909	37,550 ± 1,135	38,811 ± 1,780
6	28,938 ± 1,639	38,995 ± 951	41,079 ± 531
7	32,557 ± 896	39,668 ± 1,828	39,377 ± 953

‡ Mean counts per minute (CPM± SE) for unstimulated splenocytes were 283 ± 11, whereas CPM± SE for splenocytes stimulated with LPS were 36,678 ± 720.

Table C-2. Effect of *Dermacentor andersoni* salivary gland extracts, collected daily during course of engorgement on proliferative response of normal murine splenocytes to *Escherichia coli* lipopolysaccharide: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	** 63.8 ± 2.5	51.8 ± 0.2	40.0 ± 1.5
1	** 41.9 ± 1.3	22.6 ± 2.1	2.3 ± 2.9
2	** 26.1 ± 0.3	-	0.3 ± 10.7
3	** 27.9 ± 5.9	-	-
4	** 26.7 ± 2.4	-	-
5	** 27.6 ± 2.5	-	-
6	** 21.1 ± 4.5	-	-
7	* 11.2 ± 2.4	-	-

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine splenocytes stimulated with LPS.

* Percent suppression is significant at $P<0.05$, compared to the positive control, which consists of normal murine splenocytes stimulated with LPS.

Table C-3. Effect of *Amblyomma americanum* salivary gland extracts, collected daily during course of engorgement on proliferative response of normal murine splenocytes to *Escherichia coli* lipopolysaccharide: Mean counts per minute (CPM ± SE) ‡

	Salivary gland extract concentration		
	20 μg/ml	10 μg/ml	5 μg/ml
Day of feeding	CPM ± SE	CPM ± SE	CPM ± SE
0	13,482 ± 564	17,318 ± 303	17,960 ± 970
1	9,076 ± 208	15,440 ± 784	18,167 ± 246
2	17,391 ± 632	21,304 ± 1,255	25,122 ± 1,486
3	17,774 ± 842	22,431 ± 339	27,296 ± 946
4	22,938 ± 698	28,966 ± 455	35,235 ± 521
5	27,853 ± 1,371	35,257 ± 169	43,709 ± 941
6	22,507 ± 1,919	33,735 ± 649	38,738 ± 935
7	28,212 ± 882	35,545 ± 1,660	39,842 ± 1,262

‡ Mean counts per minute (CPM± SE) for unstimulated splenocytes were 283 ± 11, whereas CPM± SE for splenocytes stimulated with LPS were 36,678 ± 720.

Table C-4. Effect of *Amblyomma americanum* salivary gland extracts, collected daily during course of engorgement on proliferative response of normal murine splenocytes to *Escherichia coli* lipopolysaccharide: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	** 63.2 ± 1.5	52.8 ± 0.8	51.0 ± 2.6
1	** 75.3 ± 0.6	57.9 ± 2.1	50.5 ± 0.7
2	** 52.6 ± 1.7	46.2 ± 2.8	35.9 ± 0.7
3	** 51.5 ± 2.3	38.8 ± 0.9	25.6 ± 2.6
4	** 37.5 ± 1.9	21.0 ± 1.2	3.9 ± 1.4
5	** 24.1 ± 3.7	3.9 ± 0.5	-
6	** 38.6 ± 5.2	8.0 ± 1.8	-
7	** 23.1 ± 2.4	3.1 ± 4.5	-

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine splenocytes stimulated with LPS.

Table C-5. Effect of *Rhipicephalus sanguineus* salivary gland extracts, collected daily during course of engorgement on proliferative response of normal murine splenocytes to *Escherichia coli* lipopolysaccharide: Mean counts per minute (CPM ± SE) ‡

	Salivary gland extract concentration		
	20 μg/ml	10 μg/ml	5 μg/ml
Day of feeding	CPM ± SE	CPM ± SE	CPM ± SE
0	33,567 ± 796	32,141 ± 1,415	32,311 ± 445
1	28,595 ± 389	28,708 ± 976	29,240 ± 1,165
2	30,375 ± 413	30,522 ± 672	33,781 ± 1,300
3	29,175 ± 1,987	30,544 ± 725	35,103 ± 686
4	37,478 ± 1,189	40,483 ± 254	47,826 ± 1,254
5	36,091 ± 862	42,714 ± 2,223	51,183 ± 1,346
6	32,937 ± 1,168	40,011 ± 595	48,292 ± 1,602
7	36,672 ± 1,196	44,410 ± 1,367	51,071 ± 1,199

‡ Mean counts per minute (CPM± SE) for unstimulated splenocytes were 283 ± 11, whereas CPM± SE for splenocytes stimulated with LPS were 36,678 ± 720.

Table C-6. Effect of *Rhipicephalus sanguineus* salivary gland extracts, collected daily during course of engorgement on proliferative response of normal murine splenocytes to *Escherichia coli* lipopolysaccharide: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	8.5 ± 2.2	12.4 ± 3.9	11.9 ± 1.2
1	** 22.0 ± 1.1	21.7 ± 2.7	20.3 ± 3.2
2	** 17.2 ± 1.1	19.1 ±1.0	12.1 ± 1.0
3	** 20.5 ± 5.4	16.7 ± 2.0	4.3 ± 1.9
4	-	-	-
5	1.6 ± 2.4	-	-
6	* 10.2 ± 3.2	-	-
7	0.0 ± 3.3	-	-

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine splenocytes stimulated with LPS.

* Percent suppression is significant at $P<0.05$, compared to the positive control, which consists of normal murine splenocytes stimulated with LPS.

APPENDIX D

Mean values ± SE for lymphocyte blastogenesis and cytokine assays with 3 concentrations of salivary gland extracts for *D. andersoni*, *A. americanum* and *R. sanguineus* Tumor Necrosis Factor-α Assays

Table D-1. Effect of *Dermacentor andersoni* salivary gland extracts, collected daily during course of engorgement on *Escherichia coli* lipopolysaccharide-induced production of tumor necrosis factor-α by murine macrophages: Mean percent cytotoxicity ± SE ††

	Salivary gland extract concentration		
	20 μg/ml	10 μg/ml	5 μg/ml
Day of feeding	% Cytotoxicity ± SE	% Cytotoxicity ± SE	% Cytotoxicity ± SE
0	6.3 ± 5.6	28.3 ± 3.0	38.3 ± 0.9
1	26.1 ± 3.0	41.8 ± 0.9	41.4 ± 2.2
2	26.8 ± 0.9	31.2 ± 2.0	28.3 ± 1.3
3	16.0 ± 2.4	19.6 ± 4.3	23.8 ± 2.7
4	40.6 ± 0.9	33.7 ± 0.5	32.3 ± 1.5
5	32.7 ± 2.7	33.7 ± 2.1	36.8 ± 0.5
6	34.7 ± 1.2	36.4 ± 1.0	31.3 ± 4.4
7	32.8 ± 2.3	34.0 ± 1.8	31.9 ± 1.2

†† Mean % Cytotoxicity ± SE for macrophages stimulated with LPS was 59.2 ± 1.3, whereas unstimulated macrophages were not cytotoxic to macrophages.

Table D-2. Effect of *Dermacentor andersoni* salivary gland extracts, collected daily during course of engorgement on *Escherichia coli* lipopolysaccharide-induced production of tumor necrosis factor-α by murine macrophages: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 μg/ml	10 μg/ml	5 μg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	** 88.4 ± 10.2	48.1 ± 5.6	29.8 ± 1.7
1	** 52.2 ± 5.5	23.4 ± 1.7	24.1 ± 4.0
2	** 50.8 ± 1.6	42.9 ± 3.7	48.2 ± 2.4
3	** 70.7 ± 4.4	64.1 ± 7.9	56.4 ± 4.9
4	** 25.6 ± 1.6	38.3 ± 0.8	40.8 ± 2.8
5	** 40.1 ± 5.0	38.3 ± 3.8	32.6 ± 1.0
6	** 36.5 ± 2.1	33.2 ± 1.8	42.7 ± 8.0
7	** 39.9 ± 4.1	37.7 ± 3.3	41.6 ± 2.1

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine peritoneal macrophages stimulated with LPS.

Table D-3. Effect of *Amblyomma americanum* salivary gland extracts, collected daily during course of engorgement on *Escherichia coli* lipopolysaccharide-induced production of tumor necrosis factor-α by murine macrophages: Mean percent cytotoxicity ± SE ††

	Salivary gland extract concentration		
	20 μg/ml	10 μg/ml	5 μg/ml
Day of feeding	% Cytotoxicity ± SE	% Cytotoxicity ± SE	% Cytotoxicity ± SE
0	51.6 ± 1.7	57.1 ± 1.0	55.9 ± 1.3
1	58.8 ± 1.2	62.0 ± 0.3	62.7 ± 1.4
2	47.6 ± 1.9	48.3 ± 0.7	48.4 ± 3.1
3	45.4 ± 2.7	45.0 ± 4.2	52.5 ± 0.9
4	44.9 ± 2.8	43.3 ± 2.4	45.4 ± 0.9
5	33.8 ± 4.0	42.8 ± 3.3	39.8 ± 6.0
6	51.6 ± 1.5	56.5 ± 1.9	57.1 ± 2.6
7	58.1 ± 0.6	57.0 ± 3.3	59.6 ± 1.7

†† Mean % Cytotoxicity ± SE for macrophages stimulated with LPS was 59.2 ± 1.3, whereas unstimulated macrophages were not cytotoxic to macrophages.

Table D-4. Effect of *Amblyomma americanum* salivary gland extracts, collected daily during course of engorgement on *Escherichia coli* lipopolysaccharide-induced production of tumor necrosis factor-α by murine macrophages: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	** 16.9 ± 2.7	8.0 ± 1.6	10.0 ± 2.0
1	5.2 ± 1.9	0.2 ± 0.5	-
2	** 23.3 ± 3.1	22.2 ± 1.1	22.0 ± 4.9
3	** 26.9 ± 4.3	27.5 ± 6.7	15.5 ± 1.4
4	** 27.6 ± 4.5	30.2 ± 3.9	26.9 ± 1.4
5	** 45.5 ± 6.4	31.0 ± 5.3	35.9 ± 9.7
6	** 16.9 ± 2.4	8.9 ± 3.1	8.0 ± 4.3
7	6.5 ± 1.0	8.2 ± 5.3	4.0 ± 2.7

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine peritoneal macrophages stimulated with LPS.

Table D-5. Effect of *Rhipicephalus sanguineus* salivary gland extracts, collected daily during course of engorgement on *Escherichia coli* lipopolysaccharide-induced production of tumor necrosis factor-α by murine macrophages: Mean percent cytotoxicity ± SE ††

	Salivary gland extract concentration		
	20 μg/ml	10 μg/ml	5 μg/ml
Day of feeding	% Cytotoxicity ± SE	% Cytotoxicity ± SE	% Cytotoxicity ± SE
0	54.7 ± 2.5	47.9 ± 3.0	49.2 ± 1.7
1	57.0 ± 3.2	55.4 ± 1.0	54.7 ± 1.5
2	50.5 ± 4.3	47.4 ± 5.8	51.8 ± 4.2
3	53.6 ± 2.1	53.3 ± 3.8	54.1 ± 5.0
4	59.0 ± 1.8	57.3 ± 0.6	55.8 ± 1.4
5	52.9 ± 2.2	57.6 ± 1.4	54.0 ± 1.7
6	59.6 ± 2.5	60.4 ± 0.2	58.7 ± 0.2
7	55.6 ± 2.2	60.0 ± 1.8	61.5 ± 1.2

†† Mean % Cytotoxicity ± SE for macrophages stimulated with LPS was 59.2 ± 1.3, whereas unstimulated macrophages were not cytotoxic to macrophages.

Table D-6. Effect of *Rhipicephalus sanguineus* salivary gland extracts, collected daily during course of engorgement on *Escherichia coli* lipopolysaccharide-induced production of tumor necrosis factor-α by murine macrophages: Mean percent suppression ± SE:

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	* 10.1 ± 4.2	21.3 ± 4.9	19.2 ± 2.8
1	6.2 ± 5.3	8.9 ± 1.7	10.1 ± 2.5
2	* 16.9 ± 7.0	22.1 ± 9.5	14.8 ± 6.9
3	* 11.8 ± 3.5	12.3 ± 6.3	11.0 ± 8.2
4	3.0 ± 3.0	5.8 ± 0.9	8.2 ± 2.4
5	** 13.0 ± 3.6	5.3 ± 2.3	11.3 ± 2.8
6	2.0 ± 4.1	0.8 ± 0.3	3.5 ± 0.3
7	8.5 ± 3.6	1.4 ± 2.9	-

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine peritoneal macrophages stimulated with LPS.

* Percent suppression is significant at $P<0.05$, compared to the positive control, which consists of normal murine peritoneal macrophages stimulated with LPS.

APPENDIX E

Mean values ± SE for lymphocyte blastogenesis and cytokine assays with 3 concentrations of salivary gland extracts for *D. andersoni*, *A. americanum* and *R. sanguineus*
Interleukin-1 Assays

Table E-1. Effect of *Dermacentor andersoni* salivary gland extracts, collected daily during course of engorgement on *Escherichia coli* lipopolysaccharide-induced production of interleukin-1 by murine macrophages: Mean counts per minute (CPM ± SE) ‡‡

	Salivary gland extract concentration		
	20 μg/ml	10 μg/ml	5 μg/ml
Day of feeding	CPM ± SE	CPM ± SE	CPM ± SE
0	1,604 ± 97	2,839 ± 57	3,426 ± 302
1	390 ± 38	1,501 ± 86	3,574 ± 186
2	235 ± 23	825 ± 90	1,980 ± 175
3	509 ± 15	1,580 ± 116	3,059 ± 298
4	848 ± 33	2,384 ± 228	3,438 ± 89
5	1,092 ± 146	1,996 ± 50	3,321 ± 159
6	969 ± 96	2,617 ± 71	3,127 ± 76
7	879 ± 65	1,983 ± 128	2,663 ± 317

‡‡ Mean counts per minute (CPM± SE) for thymocytes stimulated with con-A (1μg/ml) were 3,579 ± 114, and with *E. coli* LPS (2μg/ml) were 123 ± 9. Mean CPM± SE for thymocytes cultured with supernatant collected from LPS-stimulated macrophages were 6,735 ± 307, whereas unstimulated macrophage-derived supernatant resulted in counts of 1,433 ± 61.

Table E-2. Effect of *Dermacentor andersoni* salivary gland extracts, collected daily during course of engorgement on *Escherichia coli* lipopolysaccharide-induced production of interleukin-1 by murine macrophages: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	** 76.2 ± 1.4	57.8 ± 0.9	49.1 ± 4.5
1	** 94.2 ± 0.6	77.7 ± 1.3	46.9 ± 2.8
2	** 96.5 ± 0.3	87.8 ± 1.3	70.6 ± 2.6
3	** 92.4 ± 0.2	76.5 ± 1.7	54.6 ± 4.4
4	** 87.4 ± 0.5	64.6 ± 3.4	48.9 ± 1.3
5	** 83.8 ± 2.2	70.4 ± 0.7	50.7 ± 2.4
6	** 85.6 ± 1.4	61.1 ± 1.1	53.6 ± 1.1
7	** 87.0 ± 1.0	70.6 ± 1.9	60.5 ± 4.7

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine peritoneal macrophages stimulated with LPS.

Table E-3. Effect of *Amblyomma americanum* salivary gland extracts, collected daily during course of engorgement on *Escherichia coli* lipopolysaccharide-induced production of interleukin-1 by murine macrophages: Mean counts per minute (CPM ± SE) ‡‡

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	CPM ± SE	CPM ± SE	CPM ± SE
0	1,065 ± 132	2,059 ± 44	3,285 ± 156
1	3,463 ± 322	4,304 ± 1,034	4,937 ± 248
2	2,648 ± 54	6,608 ± 507	5,209 ± 52
3	3,097 ± 407	3,059 ± 131	4,940 ± 354
4	3,476 ± 194	5,727 ± 448	7,450 ± 473
5	2,640 ± 95	4,220 ± 162	4,080 ± 230
6	2,734 ± 291	4,031 ± 137	4,078 ± 208
7	1,755 ± 193	2,781 ± 628	4,723 ± 280

‡‡ Mean counts per minute (CPM± SE) for thymocytes stimulated with con-A (1µg/ml) were 3,579 ± 114, and with *E. coli* LPS (2µg/ml) were 123 ± 9. Mean CPM± SE for thymocytes cultured with supernatant collected from LPS-stimulated macrophages were 6,735 ± 307, whereas unstimulated macrophage-derived supernatant resulted in counts of 1,433 ± 61.

Table E-4. Effect of *Amblyomma americanum* salivary gland extracts, collected daily during course of engorgement on *Escherichia coli* lipopolysaccharide-induced production of interleukin-1 by murine macrophages: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	** 84.2 ± 2.0	69.4 ± 0.7	51.2 ± 2.3
1	** 48.6 ± 4.8	36.1 ± 15.4	26.7 ± 3.7
2	** 60.7 ± 0.8	1.9 ± 7.5	22.7 ± 0.8
3	** 54.0 ± 6.0	54.6 ± 1.9	26.6 ± 5.3
4	** 48.4 ± 2.9	15.0 ± 6.7	-
5	** 60.8 ± 1.4	37.3 ± 2.4	39.4 ± 3.4
6	** 59.4 ± 4.3	40.1 ± 2.0	39.4 ± 3.1
7	** 73.9 ± 2.9	58.7 ± 9.3	29.9 ± 4.2

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine peritoneal macrophages stimulated with LPS.

Table E-5. Effect of *Rhipicephalus sanguineus* salivary gland extracts, collected daily during course of engorgement on *Escherichia coli* lipopolysaccharide-induced production of interleukin-1 by murine macrophages: Mean counts per minute (CPM ± SE) ‡‡

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	CPM ± SE	CPM ± SE	CPM ± SE
0	4,181 ± 115	7,039 ± 186	7,124 ± 284
1	4,329 ± 128	4,952 ± 394	7,047 ± 150
2	4,872 ± 144	7,638 ± 485	9,396 ± 144
3	5,389 ± 85	9,023 ± 323	10,154 ± 681
4	3,606 ± 200	6,773 ± 207	6,248 ± 384
5	2,641 ± 101	7,084 ± 182	7,544 ± 457
6	10,383 ± 457	10,387 ± 359	11,615 ± 612
7	8,195 ± 164	10,930 ± 530	12,174 ± 875

‡‡ Mean counts per minute (CPM± SE) for thymocytes stimulated with con-A (1µg/ml) were 3,579 ± 114, and with *E. coli* LPS (2µg/ml) were 123 ± 9. Mean CPM± SE for thymocytes cultured with supernatant collected from LPS-stimulated macrophages were 6,735 ± 307, whereas unstimulated macrophage-derived supernatant resulted in counts of 1,433 ± 61.

Table E-6. Effect of *Rhipicephalus sanguineus* salivary gland extracts, collected daily during course of engorgement on *Escherichia coli* lipopolysaccharide-induced production of interleukin-1 by murine macrophages: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	** 37.9 ± 1.7	-	-
1	** 35.7 ± 1.9	26.5 ± 5.9	-
2	** 27.7 ± 2.1	-	-
3	** 20.0 ± 1.3	-	-
4	** 46.5 ± 3.0	-	7.2 ± 5.7
5	** 60.8 ± 1.5	-	-
6	-	-	-
7	-	-	-

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine peritoneal macrophages stimulated with LPS.

* Percent suppression is significant at $P<0.05$, compared to the positive control, which consists of normal murine peritoneal macrophages stimulated with LPS.

APPENDIX F

Mean values ± SE for lymphocyte blastogenesis and cytokine assays with 3 concentrations of salivary gland extracts for *D. andersoni*, *A. americanum* and *R. sanguineus*
Interleukin-2 Assays

Table F-1. Effect of *Dermacentor andersoni* salivary gland extracts, collected daily during course of engorgement on concanavalin A-induced production of interleukin-2 by murine splenocytes: Mean counts per minute (CPM ± SE) ‡†

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	CPM ± SE	CPM ± SE	CPM ± SE
0	4,870 ± 363	5,158 ± 136	5,052 ± 147
1	5,187 ± 103	5,763 ± 111	5,704 ± 46
2	5,088 ± 152	5,571 ± 51	5,575 ± 83
3	5,351 ± 248	5,457 ± 144	5,379 ± 196
4	5,003 ± 120	5,649 ± 92	5,577 ± 88
5	5,147 ± 308	5,501 ± 30	5,409 ± 133
6	5,278 ± 134	5,710 ± 158	5,459 ± 9
7	5,626 ± 152	5,581 ± 314	5,628 ± 76

‡† Mean counts per minute (CPM ± SE) for unstimulated CTLL-2 cells were 121 ± 8, for unstimulated splenocytes were 214± 10, whereas CPM ± SE con-A stimulated splenocytes were 6,010 ± 92

Table F-2. Effect of *Dermacentor andersoni* salivary gland extracts, collected daily during course of engorgement on concanavalin A-induced production of interleukin-2 by murine splenocytes: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	** 19.0 ± 6.0	21.5 ± 2.9	14.2 ± 2.3
1	** 13.7 ± 1.7	8.4 ± 6.3	4.1 ± 1.9
2	** 15.3 ± 2.5	12.3 ± 2.7	7.3 ± 0.9
3	* 11.0 ± 4.1	13.5 ± 4.2	9.2 ± 2.4
4	** 16.8 ± 2.0	12.5 ± 3.6	6.0 ± 1.5
5	** 14.4 ± 5.1	16.7 ± 6.4	8.5 ± 0.5
6	** 12.1 ± 2.2	10.2 ± 3.4	5.0 ± 2.6
7	6.4 ± 2.5	13.7 ± 3.2	7.1 ± 5.2

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine splenocytes stimulated with Con A.

* Percent suppression is significant at $P<0.05$, compared to the positive control, which consists of normal murine splenocytes stimulated with Con A.

Table F-3. Effect of *Amblyomma americanum* salivary gland extracts, collected daily during course of engorgement on concanavalin A-induced production of interleukin-2 by murine splenocytes: Mean counts per minute (CPM ± SE) ‡†

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	CPM ± SE	CPM ± SE	CPM ± SE
0	5,077 ± 232	6,487 ± 210	7,036 ± 311
1	5,642 ± 124	6,391 ± 132	6,503 ± 119
2	5,936 ± 232	6,452 ± 372	6,562 ± 202
3	6,171 ± 66	6,653 ± 280	5,972 ± 146
4	6,106 ± 256	6,651 ± 234	6,373 ± 15
5	6,253 ± 73	7,275 ± 122	6,670 ± 26
6	6,650 ± 35	6,863 ± 257	6,827 ± 275
7	4,004 ± 104	6,972 ± 13	7,171 ± 326

‡† Mean counts per minute (CPM ± SE) for unstimulated CTLL-2 cells were 121 ± 8, for unstimulated splenocytes were 214± 10, whereas CPM ± SE con-A stimulated splenocytes were 6,010 ± 92

Table F-4. Effect of *Amblyomma americanum* salivary gland extracts, collected daily during course of engorgement on concanavalin A-induced production of interleukin-2 by murine splenocytes: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	** 15.5 ± 3.9	6.8 ± 11.9	-
1	6.1 ± 2.1	-	-
2	1.2 ± 3.9	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	** 33.4 ± 1.7	7.8 ± 19.1	-

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine splenocytes stimulated with Con A.

Table F-5. Effect of *Rhipicephalus sanguineus* salivary gland extracts, collected daily during course of engorgement on concanavalin A-induced production of interleukin-2 by murine splenocytes: Mean counts per minute (CPM ± SE) ‡†

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	CPM ± SE	CPM ± SE	CPM ± SE
0	5,814 ± 77	5,842 ± 156	5,913 ± 197
1	5,558 ± 194	5,790 ±192	5,876 ± 197
2	5,637 ± 58	5,688 ± 15	6,385 ± 40
3	5,926 ± 129	5,450 ± 125	5,621 ± 184
4	5,717 ± 207	5,942 ± 71	5,903 ± 79
5	5,658 ± 348	6,292 ± 163	6,459 ± 116
6	5,509 ± 220	5,927 ± 228	6,537 ± 213
7	5,989 ± 226	6,033 ± 92	5,872 ± 288

‡† Mean counts per minute (CPM ± SE) for unstimulated CTLL-2 cells were 121 ± 8, for unstimulated splenocytes were 214± 10, whereas CPM ± SE con-A stimulated splenocytes were 6,010 ± 92

Table F-6. Effect of *Rhipicephalus sanguineus* salivary gland extracts, collected daily during course of engorgement on concanavalin A-induced production of interleukin-2 by murine splenocytes: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	3.3 ± 1.3	5.4 ± 2.0	2.8 ± 2.6
1	7.5 ± 3.2	11.9 ± 1.5	3.7 ± 3.2
2	6.2 ±1.0	5.7 ± 0.6	5.4 ± 0.3
3	1.4 ± 2.1	3.5 ± 1.5	9.3 ± 2.1
4	4.9 ± 3.4	-	1.1 ± 1.2
5	5.9 ± 5.8	-	-
6	* 8.3 ± 3.7	0.5 ± 1.1	1.4 ± 3.8
7	0.4 ± 3.8	-	-

* Percent suppression is significant at $P<0.05$, compared to the positive control, which consists of normal murine splenocytes stimulated with Con A.

APPENDIX G

Mean values ± SE for lymphocyte blastogenesis and cytokine assays with 3 concentrations of salivary gland extracts for *D. andersoni*, *A. americanum* and *R. sanguineus*
Interleukin-4 Assays

Table G-1. Effect of *Dermacentor andersoni* salivary gland extracts, collected daily during course of engorgement on concanavalin A-induced production of interleukin-4 by murine splenocytes: Mean counts per minute (CPM ± SE) †‡

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	CPM ± SE	CPM ± SE	CPM ± SE
0	10,587 ± 1,372	15,364 ± 817	17,113 ± 267
1	6,834 ± 60	17,425 ± 1,706	19,346 ± 167
2	9,346 ± 111	14,098 ± 85	18,472 ± 507
3	11,764 ± 238	16,579 ± 658	19,898 ± 471
4	8,934 ± 219	11,850 ± 673	16,266 ± 439
5	8,688 ± 145	11,895 ± 350	16,082 ± 387
6	11,115 ± 486	15,256 ± 575	17,089 ± 464
7	11,374 ± 212	15,401 ± 596	18,995 ± 353

†‡ Mean counts per minute (CPM ± SE) for unstimulated CT-4S cells were 409 ± 19, for unstimulated splenocytes were 388 ± 15, whereas CPM ± SE con-A stimulated splenocytes were 23,238 ± 355.

Table G-2. Effect of *Dermacentor andersoni* salivary gland extracts, collected daily during course of engorgement on concanavalin A-induced production of interleukin-4 by murine splenocytes: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	** 54.4 ± 5.9	42.2 ± 3.6	33.8 ± 3.5
1	** 70.6 ± 0.3	40.5 ± 24.4	25.0 ± 7.3
2	** 59.8 ± 0.5	49.1 ± 8.0	39.3 ± 0.4
3	** 49.4 ± 1.0	40.2 ± 5.9	28.7 ± 2.8
4	** 61.6 ± 0.9	56.6 ± 2.8	49.0 ± 2.9
5	** 62.6 ± 0.6	57.5 ± 4.7	48.8 ± 1.5
6	** 52.2 ± 2.1	40.1 ± 8.6	34.4 ± 2.5
7	** 51.1 ± 0.9	40.9 ± 7.0	33.7 ± 2.6

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine splenocytes stimulated with Con A.

Table G-3. Effect of *Amblyomma americanum* salivary gland extracts, collected daily during course of engorgement on concanavalin A-induced production of interleukin-4 by murine splenocytes: Mean counts per minute (CPM ± SE) †‡

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	CPM ± SE	CPM ± SE	CPM ± SE
0	13,566 ± 222	17,637 ± 476	18,422 ± 1060
1	16,586 ± 346	18,403 ± 1,055	21,486 ± 324
2	22,195 ± 244	24,243 ± 685	23,815 ± 276
3	19,406 ± 265	23,434 ± 544	23,507 ± 494
4	9,079 ± 111	19,930 ± 492	23,561 ± 135
5	4,510 ± 79	14,678 ± 596	22,603 ± 431
6	5,847 ± 177	16,837 ± 91	21,201 ± 125
7	1,454 ± 15	9,963 ± 270	17,713 ± 249

†‡ Mean counts per minute (CPM ± SE) for unstimulated CT-4S cells were 409 ± 19, for unstimulated splenocytes were 388 ± 15, whereas CPM ± SE con-A stimulated splenocytes were 23,238 ± 355.

Table G-4. Effect of *Amblyomma americanum* salivary gland extracts, collected daily during course of engorgement on concanavalin A-induced production of interleukin-4 by murine splenocytes: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	** 41.6 ± 1.0	33.1 ± 7.2	24.1 ± 2.1
1	** 28.6 ± 1.5	24.5 ± 3.2	20.8 ± 4.5
2	4.5 ± 1.0	-	-
3	** 16.5 ± 1.1	11.2 ± 6.1	-
4	** 60.9 ± 0.5	38.5 ± 17.6	14.2 ± 2.1
5	** 80.6 ± 0.3	61.5 ± 16.0	36.8 ± 2.6
6	** 74.8 ± 0.8	52.0 ± 19.8	27.5 ± 0.4
7	3.7 ± 0.1	74.4 ± 15.9	57.1 ± 1.2

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine splenocytes stimulated with Con A.

Table G-5. Effect of *Rhipicephalus sanguineus* salivary gland extracts, collected daily during course of engorgement on concanavalin A-induced production of interleukin-4 by murine splenocytes: Mean counts per minute (CPM ± SE) †‡

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	CPM ± SE	CPM ± SE	CPM ± SE
0	24,312 ± 146	25,726 ± 980	24,870 ± 482
1	20,557 ± 444	22,982 ± 872	23,684 ± 162
2	23,806 ± 785	23,614 ± 441	22,512 ± 605
3	20,051 ± 800	21,512 ± 854	21,241 ± 484
4	14,205 ± 397	17,618 ± 443	20,943 ± 418
5	11,327 ± 392	15,611 ± 312	19,823 ± 772
6	9,542 ± 251	15,773 ± 211	21,969 ± 519
7	6,050 ± 144	14,077 ± 157	19,683 ± 27

†‡ Mean counts per minute (CPM ± SE) for unstimulated CT-4S cells were 409 ± 19, for unstimulated splenocytes were 388 ± 15, whereas CPM ± SE con-A stimulated splenocytes were 23,238 ± 355.

Table G-6. Effect of *Rhipicephalus sanguineus* salivary gland extracts, collected daily during course of engorgement on concanavalin A-induced production of interleukin-4 by murine splenocytes: Mean percent suppression ± SE

	Salivary gland extract concentration		
	20 µg/ml	10 µg/ml	5 µg/ml
Day of feeding	% Suppression ± SE	% Suppression ± SE	% Suppression ± SE
0	-	-	-
1	** 11.5 ± 1.9	4.6 ± 8.3	1.1 ± 3.8
2	-	-	-
3	** 13.7 ± 3.4	11.6 ± 0.2	7.4 ± 3.7
4	** 38.9 ± 1.7	33.0 ± 4.3	24.2 ± 1.9
5	** 51.3 ± 1.7	44.8 ± 7.8	32.8 ± 1.3
6	** 58.9 ± 1.1	45.8 ± 12.0	32.1 ± 0.9
7	** 74.0 ± 0.6	57.2 ± 14.7	39.4 ± 0.7

** Percent suppression is significant at $P<0.01$, compared to the positive control, which consists of normal murine splenocytes stimulated with Con A.

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VITA

Mona Asad Abdallah

Candidate for the Degree of

Doctor of Philosophy

Thesis: COMPARISON OF THE IMMUNOSUPPRESSIVE ABILITY OF SALIVARY GLAND EXTRACTS OF THE IXODID TICKS: *DERMACENTOR ANDERSONI, AMBLYOMMA AMERICANUM* AND *RHIPICEPHALUS SANGUINEUS*

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