INFLUENCE OF THE IXODID TICK, *DERMACENTOR ANDERSONI*, ON MURINE ADHESION MOLECULES, CYTOKINES, AND LYMPHOCYTE RESPONSES

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NOMENCLATURE

ADP	adenosine monophosphate
ADP	adenosine diphosphate
BCA	bicinchoninic acid
bEnd3	capillary endothelial cells derived from brain tissue
BSA	bovine serum albumin
CAM	cellular adhesion molecule
CBH	cutaneous basophil hypersensitivity
$CD4^+$	cells positive for cluster if differentiation designation 4 (helper T lymphocytes)
$CD8^+$	cells positive for cluster if differentiation designation 8 (cytotoxic T lymphocytes)
CLA	cutaneous lymphocyte antigen
CPM	counts per minute
Con A	concanavalin A
DMEM	Dulbecco's Modified Eagle Medium
ELISA	enzyme-linked immunosorbant assay
FBS	fetal bovine serum
HEV	high endothelial venules
HRPO	horseradish peroxidase

ICAM-1	intercellular adhesion molecule-1
IFN	interferon
Ig-CAM	immunoglobulin-like cellular adhesion molecule
IL	interleukin
LFA-1	lymphocyte function-related antigen-1
LPS	lipopolysaccharide
MBP	mannan-binding lectin
MHC	major histocompatibility complex
mrTNF-α	murine recombinant tumor necrosis factor-α
NK	natural killer cells
OD	optical density
OPD	o-phenylenediamine-dihydrochloride
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PRRs	pattern recognition receptors
S.E.M.	standard error of the mean
SI	stimulation index
SGE	salivary gland extract
Th	T helper lymphocytes
TNF	tumor necrosis factor
VCAM-1	vascular cellular adhesion molecule-1
VLA-4	very late activation-4

CHAPTER I

PERSPECTIVES AND OVERVIEW

Introduction

Ticks and tick-borne diseases are increasingly important global public health concerns (Walker, 1998). Ticks are the most important arthropod involved in transmission of pathogens to animal species other than humans, and they are second only to mosquitoes as vectors of disease-causing agents to humans (Balashov, 1972). In the United States, there are a number of ticks of medical importance, including; *Dermacentor andersoni*, vector of *Rickettsia rickettsii*, Colorado tick fever virus, and *Francisella tularensis*; *D. variabilis*, vector of *R. rickettsii* and *F. tularensis*; *Amblyomma americanum*, vector of *Ehrlichia chaffeensis* and *F. tularensis*; and, *Ixodes scapularis*, vector of *Borrelia burgdorferi*, *E. equi*, and *Babesia microti* (Walker, 1998). Although the incidence of some vector borne-diseases had declined, in recent years, there has been an overall resurgence of these diseases worldwide (Gratz, 1999). Factors responsible for the emergence or resurgence of vector-borne diseases include insecticide/acaricide resistance, drug resistance, changes in public health policy, environmental changes, mass migration of people, urbanization, and genetic changes in pathogens (Gubler, 1998; Molyneux, 1998; Wikel, 1999).

Tick-borne disease-causing agents are introduced into the host in saliva, which contains an array of pharmacologically active molecules designed to reduce host grooming and to counteract host hemostatic and immune defenses (Ribeiro, 1995; Wikel

and Bergman, 1997; Wikel, 1999). Understanding those aspects of tick biology that contribute to successful feeding and pathogen transmission will provide valuable insights for development of novel strategies for the control of both ticks and tick-transmitted diseases (Wikel and Bergman, 1997; Walker, 1998).

To better understand the complexity of the tick-host relationship, a literature review is provided. Addressed in the review will be the components of the host immune system, activation of the immune system during tick feeding, acquired resistance to ticks, and tick modulation of the host immune response. A detailed description of the molecules and mechanisms involved in leukocyte migration during an immune response will provide the basis for the current research.

Literature Review

Immunity, innate and acquired (adaptive), is the capacity to distinguish foreign material from self, and to neutralize, eliminate, or metabolize that which is foreign by the physiological mechanisms of the immune response (Medzhitov and Janeway, 1997). A dynamic relationship exists between the host defenses and the countermeasures to innate and acquired immunity by disease causing agents.

Innate immunity: Innate immunity is resistance that is not acquired through previous contact with an antigen (Robertson, 1998). Considered the primary line of defense, it is non-specific and includes natural barriers to infectious organisms. Other aspects of innate immunity are host defense mechanisms that exist in the sub-epithelium, which are responsible for removing pathogens that cross the epithelial barrier.

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The epithelia that comprise skin and the linings of the body's tubular structures provide mechanical, chemical, and microbiological barriers to infection (Fearon and Locksley, 1996). Mechanical aspects include epithelial cells joined by tight junctions and the movement of mucus by cilia, which prevents bacteria from colonizing on the epithelial surface. Lysozyme, which is present in saliva, sweat, and tears, and fatty acids of the skin are examples of chemical barriers. Normal flora, that compete for nutrients and attachment sites on epithelium, can also produce antibacterial proteins that provide anti-microbial protection (Janeway and Travers, 1997).

Microorganisms that overcome the epithelial barrier are subject to host defense mechanisms. Considered an innate humoral response, the activation of complement through either the alternative or lectin pathways will result in a cascade of reactions that will destroy invading microorganisms (Robertson, 1998). The alternative pathway of complement is activated by the binding of C3b to the surface of a microorganism. Two acute phase proteins, C-reactive protein and mannan-binding lectin, produced by the liver, are capable of activating the classical pathway and lectin pathway, respectively, of complement. The C-reactive protein recognizes the conserved phosphorylcholine component of gram-negative bacteria and binds the C1q component of complement (Robertson, 1998). Mannan-binding lectin (MBP) recognizes bacterial cell surfaces through their mannose-containing carbohydrates (Robertson, 1998). Furthermore, complement factors C3a, C4a, and C5a, produced during the activation of complement, act as inflammatory mediators and contribute to cellular traffic (Janeway and Travers, 1997).

Innate cellular immune responses are mediated by phagocytes. The two major phagocytes of the innate immune system are the macrophages and the neutrophils (Robertson, 1998). Macrophages, which differentiate from monocytes, are capable of trapping, engulfing, and destroying pathogens in tissue, and their secretion of cytokines is critical in all phases of host defense. Furthermore, receptors on macrophages play an important role in antigen uptake and presentation, contributing to the induction of the adaptive immune response (Fearon and Locksley, 1996). Conserved molecular structures called pathogen-associated molecular patterns (PAMPs) are shared by large groups of pathogens and are essential for the physiology and survival of microorganisms (Janeway, 1992). PAMPs include teichoic acids and lipopolysaccharide (LPS), common to gram-positive and gram-negative bacteria, respectively; and, mannans, which are conserved components of yeast cell walls (Medzhitov and Janeway, 1997). Macrophages are able to recognize PAMPs with a variety of conserved surface receptors called pattern recognition receptors (PRRs) (Medzhitov and Janeway, 1997). In addition to PRRs, complement and scavenger receptors are also present on the surface of macrophages (Robertson, 1998).

Although neutrophils phagocytose pathogens, they are not antigen-presenting cells. Neutrophils are the predominate cellular infiltrate into inflammatory sites, which produce essential bacteriostatic and toxic substances that kill phagocytosed pathogens (Rosales and Brown, 1993). Neutrophils express PRRs, complement, and cytokine receptors on their surface, similar to that described for macrophages (Robertson, 1998). Phagocytes also release a variety of mediators; prostaglandins, oxygen radicals, peroxides, nitric oxide, leukotrines, and platelet-activating factor, in response to infection

(Hauschildt and Kleine, 1995). Because secretion of cytokines by phagocytes, particularly macrophages, is important for an induced non-adaptive response, as well as initiation of adaptive immune response, a general description of the role of cytokines in relation to these responses is appropriate.

Initiation and maintenance of the immune response are regulated by cytokines (Fresno et al., 1997). Cytokines are secreted by particular cell types in response to a variety of stimuli and produce characteristic effects on growth, motility, differentiation, and function of target cells (Oppenheim and Ruscetti, 1997). Cytokines are broadly classified based on their role in the immune response. Categories included: growth factors (e.g. tumor growth factor-beta); modulators of leukocyte chemotaxis, also called chemokines (e.g. interleukin (IL)-5 and IL-8); modulators of lymphocyte function (e.g. IL-2 and IL-4); and, modulators of an inflammatory response (e.g. interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), IL-1, IL-6, and IL-10) (Mayers and Johnson, 1998).

Cytokines secreted by macrophages in response to infection include IL-1, IL-6, IL-8, IL-12, and TNF-α (Van der Meide and Schellekens, 1996). Kerotinocytes, which have a central role in the immune response in the epidermis, secrete the cytokines IL-1, IL-6, IL-8, IL-10, and IFN-γ (Wikel, 1996b). $CD4^+$ T cells control both the cellular and humoral (antibody-mediated) responses of acquired specific immunity by producing the cytokines IFN-γ, IL-2, IL-4, IL-5, and IL-10 (Van der Meide and Schellekens, 1996). The combined effects of specific cytokines and mediators produced by phagocytes, and to

a certain extent, the activation of complement inflammatory mediators by infectious agents contribute to inflammation.

During the initial phase of inflammation, transvascular shifts of fluid, plasma proteins, and leukocytes occur in the microvasculature of the injured or infected tissue (Tan et al., 1999). Concerted effects of IL-1, IL-6, IL-8, and TNF-α produced by macrophages induce a variety of responses, including the ability to signal bone-marrow endothelium to release neutrophils and hepatocytes to synthesize acute-phase proteins (Janeway, 1992). Neutrophils are directed to the site of inflammation by IL-8 (Mayers and Johnson, 1998). Acute-phase proteins act as opsonins, and the increased migration of neutrophils to the site augment removal of opsonized pathogens (Robertson, 1998).

Macrophages also play a critical role in the development of an acquired immune response. IL-1, IL-6, and TNF-α help activate B and T cells, which when combined with increased antigen presentation, will increase the acquired immune response (Van der Meide and Schellekens, 1996). Additionally, IL-12 activates natural killer (NK) cells to produce IFN-γ, which induces the differentiation of $CD4^+$ T cells to T helper (Th) 1 cells (Gumperez and Parham, 1995; O'Garra, 1998).

Acquired immunity: Acquired specific immunity occurs after exposure to an infectious agent, and it is characterized by activation of antigen-specific B and T cells that proliferate and differentiate into effector cells (Medzhitov and Janeway, 1997). Antigen presenting cells including; macrophages, B lymphocytes, Langerhans cells, and dendritic cells are critical to the formation of an acquired immune response (Wikel, 1996d).

Similar to innate immunity, cytokines and other molecules mediate cell differentiation and migration to sites of infection (Litchman and Abbas, 1997).

Antigen introduced into the host through the skin will come into contact with Langerhans cells or kerotinocytes present in the epidermis (Wikel, 1996d). Langerhans cells can migrate from the epidermis to the draining lymph node for presentation of antigen in the context of major histocompatibility complex class II (MHC class II) to T lymphocytes (Wikel, 1996d). Keratinocytes also express MHC class II molecules, and can present antigen and co-stimulatory signals resulting in the activation of T lymphocytes (Wikel, 1996d; Barneston et al., 1998).

Initial functional differentiation of naive $CD4^+$ T cells is influenced by the cytokines secreted in the early stages of infection (Litchman and Abbas, 1997). Naive $CD4^+$ T cells will differentiate into one of two effector T cells, Th1 or Th2. Th2 cells are activators of B cells (antibody production), while Th1 cells are crucial for macrophage activation (Mosmann and Coffman, 1989). It is generally thought that Th1 responses mediate killing of intracellular parasites, and Th2 responses eliminate extracellular parasites (Allen and Maizels, 1997). When stimulated with IL-12 and IFN-γ produced by macrophages and NK cells, $CD4^+$ T cells will differentiate into Th1 cells. In contrast, $CD4^+$ T cells activated in the presence of IL-4 and IL-6 differentiate into Th2 cells (Allen and Maizels, 1997). $CD8^+$ T cells, in addition to their cytotoxic function, can secrete cytokines typical of Th1 or Th2 cells (Romagnani, 1997).

The type of T cell response generated is crucial in determining whether the specific acquired immune response will be predominately cell mediated or antibody

mediated. In addition to the cytokines produced in the initial phases of infection, the subsets of $CD4^+$ T cells can regulate the growth and effector functions of one another. IL-10, a product of Th2 cells, can inhibit the development Th1 cells by preventing the activation and proliferation of macrophages (O'Farrell et al., 1998). Likewise, IFN-γ, a product of Th1 cells, can prevent activation of Th2 cells (O'Garra, 1998). Although distinct cytokine profiles can be associated with Th1 and Th2 phenotypes, examination of individual cytokines, and the effector pathways induced by these cytokines is necessary when assessing the immunological response (Allen and Maizels, 1997).

Throughout the inflammatory response and events associated with innate and acquired immunity is the accumulation of cells and soluble mediators at the site of an invading microorganism. Complex cellular interactions, mostly orchestrated by cytokines, result in an influx of cells to sites of infection. At these sites, leukocyte-endothelial adhesion molecule interactions are required for effective control of the infection.

Adhesion molecules: It has become well established that vascular endothelium is an important regulatory organ in immunity and homeostasis (Springer, 1990; Tan et al., 1999). In this section, descriptions of the cellular adhesion molecules (CAMs), including the cells that express specific adhesion molecules, and the influence of cytokines on these molecules, are provided.

Classification of CAMs is based on chemical, structural, or functional similarities. Some fundamental characteristics of all CAMs include; 1) they are transmembrane glycoproteins, with extracellular domains attached to a intramembranous domain, which

in turn is attached to a cytoplasmic domain; 2) activation is achieved through an external stimulus, either another cell or matrix bound molecule, and the molecule that binds the CAM is a specific receptor or ligand; 3) the intracellular domain of the molecule is attached to another molecule that induces functional changes within the cell (Freemont, 1998). Currently, CAMs, are divided into six families: the immunoglobulin-like (Ig-CAM) superfamily, selectins, integrins, cadherins, receptor protein tyrosine phosphatases, and the hyaluronate receptors. All CAMs, except for members of the hyaluronate receptors are involved in cell-cell adhesion (Freemont, 1998), and for purposes of this review, Ig-CAMs, selectins, and integrins are further described.

Members of the Ig-CAM superfamily have characteristic extracellular domains that resemble immunoglobulin (Springer, 1990). Intercellular adhesion molecule-1 (ICAM-1), ICAM-2, and vascular cellular adhesion molecule-1 (VCAM-1) are members of the Ig-CAM family and are expressed, or inducible, on vascular endothelium (Springer, 1990; 1994). ICAM-1 has five extracellular domains, is expressed at low levels on some vascular endothelium and lymphocytes, and is inducible 4-6 hours after cells are exposed to endotoxin, IL-1, or TNF-α (Etzioni, 1996). VCAM-1 has seven extracellular domains, and although expression is inducible in a pattern similar to ICAM-1, it is constitutively expressed on endothelium at a higher level than ICAM-1 (Springer, 1990).

The selectins were first described in 1989, and distinguishable by three distinct glycoproteins found on endothelium [E-selectin; (Bevilacqua et al., 1989)], platelets [P-selectin; (Johnston et al., 1989)], and leukocytes [L-selectin; (Lasky et al., 1989)]. All

three selectins have a common lectin-like domain, but they are differ in their distribution, activation, and mode of expression. Expression of E-selectin is restricted to endothelial cells, *de novo* synthesis is induced by IL-1 or TNF-α, and peak expression at 4-6 hours returns to basal levels by 24 hours (Bevilacqua, 1993). P-selectin, which is expressed on platelets and endothelial cells, is stored in granules inside the cell, and redistribution and expression on the surface can occur within two minutes of cell activation by thrombin or histamine (Etzioni, 1996). In contrast to the expression of E- and P-selectin on endothelial cells, L-selectin is only expressed on leukocytes, including lymphocytes, monocytes, and neutrophils (Bevilacqua, 1993). The function of each selectin differs as well. While P-selectin is important in mediating adhesion between leukocytes and platelets during clotting and at times of platelet activation, L- and E-selectin are important mediators of lymphocyte trafficking in lymph nodes and tissues.

Integrins are heterodimer molecules, consisting of one α chain and one β chain, involved in both cell-cell and cell-matrix adhesion (Hynes, 1987). The specificity of ligand binding is dependent on the α subunit, but both are necessary for ligand binding and functional activity, and because many of β chains are rare, further classification of integrins is based on the β subunit resulting in β1, β2, and β3 classes of integrins (Hynes, 1992; Jones and Walker, 1999). Of the three classes of β integrins, β1 and β3 are coexpressed on most cell types and are predominately involved in the interactions of cells with their matrices, while β2 integrins are expressed only on leukocytes and are involved in cell-cell adhesion (Freemont, 1998; Jones and Walker, 1999). The $\alpha_L\beta_2$ integrin, also called lymphocyte function-related antigen-1 (LFA-1), is expressed on lymphocytes,

monocytes, and neutrophils, and is important in leukocyte adhesion to activated endothelium and migration of leukocytes into tissues during and inflammatory response (Springer, 1995). Another important integrin involved in the inflammatory response is the $\alpha_4\beta_1$ integrin, very late activation-4 (VLA-4), which is expressed on lymphocytes, macrophages, eosinophils, basophils, NK cells, and monocytes, and binds to cell surface molecules expressed on activated endothelium (Springer, 1995). The interactions of the Ig-CAMs, selectins, and integrins in relation to their specific roles in cellular migration is presented in the following section.

Cellular trafficking: The circulatory and migratory properties of leukocytes allows for the effective surveillance of tissues for infectious pathogens and rapid accumulation of cells at sites of injury or infection (Springer, 1995). Neutrophils, monocytes, and lymphocytes have different functions in an immune response and consequently, have different patterns of migration. Both neutrophils and monocytes are phagocytic and accumulate at tissue sites in response to injury or bacterial infection. However, monocytes, not neutrophils, accumulate at sites in which T lymphocytes have recognized antigen and are important effector cells in T cell immunity. Because neutrophils and monocytes cannot recirculate, migration into tissue occurs only once. Additionally, after migration across the endothelium, monocytes differentiate into either longer-lived tissue macrophages or fuse to form multinucleate giant cells (Springer, 1995).

In contrast to phagocytic leukocytes, lymphocytes can recirculate through the body a number of times. Lymphocytes migrate through tissue to the lymphatics, percolate through the draining lymph nodes in the lymphatic system, enter the thoracic duct and

eventually return to the bloodstream (Springer, 1995). Depending on the type of lymphocyte, migration across either high endothelial venules (HEV) of lymph nodes (naive T cells) or vascular endothelium (effector T cells) occurs. The interactions of CAMs expressed by phagocytes and lymphocytes (in lymphoid tissue and skin) with those expressed on endothelium, and the steps in adhesion should be examined.

The endothelium also plays an active role in inflammatory responses (Bevilacqua, 1993). The localization of cells is driven by the interplay between cell-surface receptors and matrix molecule interactions that regulate adherence and chemoattractant gradients that direct cell migration (Etzioni, 1996). The adhesion of leukocytes to endothelial cells is mediated by cytokines, which facilitate specific interactions of receptor-ligand pairs, and include the selectin-carbohydrate and IgCAMs-integrin interactions (Springer, 1990; Lipsky, 1993).

As previously discussed, the first phase of host defense is innate immunity. The cellular mediated aspect of innate immunity, phagocytic activity, requires cells to enter the vascular endothelium to combat infection (Hauschildt and Kleine, 1995). The initial step of cell trafficking is the interaction of selectins induced on endothelium and their carbohydrate ligands expressed on the leukocyte. Phagocytic leukocytes express the sialyl-Lewis^x moiety (S-Le^x), which binds the ligand E-selectin on the endothelium. This interaction does not anchor the cells, but by making and breaking contact, leukocytes "roll" along the endothelium (Carlos and Harlan, 1994). During this period, stronger interactions of induced ICAM-1 on the endothelium and its receptor LFA-1 on the leukocyte facilitate tight binding of the leukocyte, and rolling is arrested. Extravasation,

migration between endothelial cells, is accomplished through interactions of LFA-1 and Mac-1 with the molecule CD31 at the intercellular junctions of endothelial cells (Springer, 1995).

Phagocytes also have receptors for the chemokine IL-8, which enables their migration into tissues to sites of infection along a concentration gradient of IL-8 (Carlos and Harlan, 1994). Similar processes account for the homing of naive T lymphocytes to lymphoid organs and effector T cells to sites of infection.

Naive T cells enter lymphoid organs essentially by the same mechanism described for entry of phagocytes into sites of infection (Springer, 1995). However, T cell interaction with HEV in lymph nodes is unique because expression of the selectin is by the T cell, rather than the endothelium. L-selectin, expressed on naive T cells, binds sulfated carbohydrates of CD34 expressed on the HEV cells of lymph nodes (Picker and Butcher, 1993). The binding of L-selectin facilitates a rolling interaction, which allows stronger interaction between LFA-1 and ICAM-1 or ICAM-2. The binding of LFA-1 to its ligands ICAM-1 and ICAM-2 plays a major role in the migration of T cells into the lymph node (Springer, 1994).

Activation of naive T cells, into armed effector T cells, is accompanied by changes in adhesion molecule expression by the cell. Most armed effector T cells lose the expression of L-selectin and increase the expression of VLA-4 (Baron et al., 1993). These changes no longer direct T cells to lymph nodes, but rather direct them to sites of infection by interactions of VLA-4 with VCAM-1 on cytokine stimulated endothelial cells in the peripheral tissues (Springer, 1995). T cells migrating to the skin will express

cutaneous lymphocyte antigen (CLA), which binds E-selectin (Fuhlbrigge et al., 1997). Lymphocytes enter the perivasculature by essentially the same steps as described for phagocytes: rolling along the endothelium, firm attachment, and extravasation through the endothelium to the site of infection (Springer, 1995). Many of the cells involved in the immune response to infection are also involved in the host response to tick feeding.

Host immune response and acquired resistance to tick feeding: A complete understanding of the host immune response to the vector, vector countermeasures to host immunity, and how these interactions impact vector transmission and establishment will be required to understand the dynamics of infestation, pathogen transmission, and for development of effective control strategies (Wikel et al., 1994; Wikel, 1999). These tick-host-pathogen immunological relationships involve an array of complex humoral and cellular interactions (Wikel, 1996a). Tick feeding stimulates host immune regulatory and effector pathways involving antigen-presenting cells, complement, granulocytes, B and T lymphocytes, homocytotropic and circulating antibodies, cytokines, and a variety of biologically active molecules (Willadsen, 1980; Wikel, 1982; 1996a; 1999). The host immune response can interfere with tick feeding in a number of ways. Molecules introduced into the host through the saliva can trigger the itch sensation, resulting in the removal of the tick by host grooming (Alexander, 1986). In some tick-host associations, the immune response elicited by the host during repeated tick exposure can facilitate the development of acquired resistance to tick feeding.

Acquired resistance to feeding is expressed as reduced engorgement weight; diminished ova production; and, death of feeding ticks (Wikel, 1996a; 1999). The

immune responses involved in the development of acquired resistance to tick infestation include; antigen-presenting cells, complement-dependent immune effector mechanisms, B and T lymphocytes, homocytotropic and circulating antibodies, cell mediated immunity, cytokines, and numerous bioreactive molecules (Wikel et al., 1994; Wikel and Bergman, 1997; Wikel, 1982; 1996a; 1999).

Langerhans cells in the epidermis can recognize tick antigen, migrate to the draining lymph nodes, and act as antigen-presenting cells to lymphocytes that are specifically reactive to tick salivary molecules (Allen et al., 1979b; Nithiuthai and Allen, 1985). In tick resistant hosts, the importance of Langerhans cells in the expression of acquired resistance has been established (Nithiuthai and Allen, 1984a; Wikel, 1996d; 1999). Guinea pigs, in which Langerhans cell were depleted by short-wavelength ultraviolet irradiation prior to initial infestation, showed reduced ability to develop acquired resistance (Nithiuthai and Allen, 1984b). Furthermore, when guinea pigs that had already developed acquired resistance to ticks were depleted of Langerhans cells, expression of acquired resistance during a challenge infestation was also reduced (Nithiuthai and Allen, 1984b).

Complement has been shown to be involved in the immune response to tick feeding. Rabbits repeatedly infested with *I. ricinus* had increased levels of complement component C3 during successive infestations (Papatheodorou and Brossard, 1987). Moreover, complement activation during tick feeding is important for the development of acquired resistance (Wikel, 1979; 1996a; 1999). *In vitro* administration of a complement depleting factor, cobra venom factor, inhibited the expression of acquired resistance in

guinea pigs that had previously developed acquired resistance to *D. andersoni* (Wikel and Allen, 1977). Complement fixation by the classical pathway involves complexes of salivary gland antigens, IgG, and complement, which were found to be localized at the dermal-epidermal junction of tick-resistant guinea pigs (Allen et al., 1979a). There is evidence that the alternative pathway of complement is also important in acquired resistance. Guinea pigs that were deficient in the complement component C4, and therefore unable to activate complement through the classical pathway, expressed acquired resistance (Wikel, 1979).

The humoral response to tick infestation is important in the development of acquired resistance (Whelen and Wikel, 1993; Wikel, 1996a; 1999). Depletion of B lymphocytes with cyclophosphamide blocked the development of acquired resistance in guinea pigs (Wikel and Allen, 1976), thus indicating a requirement of antibody for the development of acquired resistance. Antibodies, both homocytotropic and circulating, specific for tick antigens are produced in response to tick feeding (Shapiro et al., 1986; Wikel, 1999). Tick infested animals produce homocytotropic antibodies that bind Fc receptors on basophils (Brossard and Girardin, 1979; Christe et al., 1998), and through degranulation of these cells, inflammatory mediators are released (Wikel, 1999). Circulating tick-specific antibodies are also produced, and through their activation of complement, increase cellular traffic to tick attachment sites and enhance the inflammatory response (Wikel, 1999).

The immunological basis of acquired resistance to tick feeding was first reported by Trager (1939). Guinea pigs that developed cutaneous responses at tick attachment

sites also expressed acquired resistance to tick feeding, compared to hosts that were non-resistant and did not develop cutaneous responses (Trager, 1939; Wikel, 1996a). Acquired resistance to tick infestation has been correlated with development of cellular infiltrates, particularly basophils and eosinophils, at tick attachment sites on resistant hosts (Allen, 1989; Wikel, 1982; 1996a). The nature of the cutaneous histological response depends on the tick-host relationship (Wikel, 1996a). Several studies have illustrated the importance of cell mediated responses in the development of acquired resistance. Guinea pigs expressing acquired resistance to *D. andersoni* developed cutaneous basophil hypersensitivity reactions (CBH) at bite sites (Allen, 1973; Askenase, 1977), which were correlated to reduced tick feeding. High numbers of degranulated mast cells, and prominent infiltration of eosinophils, were observed at attachment sites on BALB/c mice infested repeatedly infested with *D. variabilis* larvae, suggesting a role for these cells in the expression of acquired resistance (denHollander and Allen, 1985b).

Additional support for the importance of basophils in acquired resistance was provided by studies utilizing mast cell-deficient mice, and through passive administration of anti-basophil antibodies. Mast cell-deficient mice developed acquired resistance after two infestations (denHollander and Allen, 1985b), and ultrastructural analysis revealed that large numbers of basophils had accumulated at tick attachment sites (Steeves and Allen, 1990). It is been suggested that the influx of basophils compensated for mast cell deficiency (Steeves and Allen, 1990; Wikel, 1996a). The expression of acquired resistance to *A. americanum* was not observed in guinea pigs that were administered anti-basophil serum (Brown et al., 1982).

The functions of basophils, mast cells, and eosinophils are interrelated, and the interaction of cells and molecules at tick attachment sites during feeding is important in the development of acquired resistance (Wikel, 1996c). In addition, complement activation and antibody production also contribute to the formation of the CBH response associated with acquired resistance. Activation of complement through either the alternative or classical pathway will increase levels of C5a, which as a chemoattractant, will lead to a greater accumulation of basophils (Wikel, 1996a; 1999).

As discussed earlier, tick-specific homocytotropic antibodies occupy Fc receptors of resident mast cells and infiltrating basophils, contributing to the degranulation of these cells during feeding (Brossard and Fivaz, 1982; Christe et al., 1998). Degranulation of basophils and mast cells will result in the release of chemotactic molecules like histamine, which in turn, will signal more eosinophils to tick attachment sites (Wikel and Bergman, 1997; Wikel, 1999). In addition to cell signaling, histamine was shown to modify *D. andersoni* feeding by reducing salivation and engorgement behavior *in vitro* (Paine et al., 1983). Histamine also induces changes in vascular endothelium, allowing for an increase in cellular traffic to tick attachment sites (Springer, 1995).

T lymphocytes play a central role in the regulation of host immune responses to ticks, and the differentiation of T lymphocytes in response to tick infestation is important to the development of acquired resistance (Wikel, 1996a; 1999). Salivary gland extracts from partially fed female *D. andersoni* induced *in vitro* proliferation of lymphocytes recovered from guinea pigs that had been previously infested with ticks (Wikel et al., 1978), indicating that recognition of tick antigen by lymphocytes does occur in the host.

T lymphocyte recognition of tick antigen will result in T cell differentiation and production of cytokines (Wikel, 1999).

The $CD4^+$ T cell subsets, Th1 and Th2, have immunoregulatory and effector functions in acquired resistance (Wikel, 1996a; 1999). Th1 cells mediate delayed hypersensitivity reactions, which are effectors of the influx of basophils previously described (Mosmann and Coffman, 1989); and, Th2 cells are responsible for the immediate hypersensitivity component of acquired resistance by the development of homocytotropic antibodies (Wikel and Bergman, 1997; Wikel, 1999). The phenotype of infiltrating T lymphocytes of BALB/c mice infested with *I. ricinus* ticks at attachment sites was determined using immunohistochemical analysis (Mbow et al., 1994b). The mice exhibited a two-fold increase in the number of $CD4^+$ T cells, compared to $CD8^+$ T cells, during the primary infestation. During secondary and tertiary infestations, there were 3.2 and 4.7 - fold increases in $CD4^+$ T cells, respectively, compared to $CD8^+$ T cells. During all three infestations, the number of infiltrating T lymphocytes, $CD4^+$ and $CD8^+$, was greater than that observed for control mice. Furthermore, B lymphocytes were not detected in the skin of either control or infested mice during any of the three successive infestations (Mbow et al., 1994b). Despite the marked increase in both $CD4^+$ and $CD8^+$ T cells, BALB/c mice failed to develop acquired resistance to tick feeding (Mbow et al., 1994a).

Cytokine production is also enhanced in response to tick feeding (Wikel, 1996a; 1996b; 1999). The presence of mRNA of various cytokines in cells infiltrating the skin, and in draining lymph nodes, was determined for BALB/c mice repeatedly infested with

I. ricinus nymphs using immunostaining and *in situ* hybridization (Mbow et al., 1994c; Mbow et al., 1994b). Cells containing IFN-γ and IL-2, but minimal IL-4 mRNA, were detected in lymph node sections 72 hours after primary infestation, and this pattern remained consistent through secondary and tertiary infestations (Mbow et al., 1994c). In skin biopsies, less than five percent of the infiltrating cells were positive for IFN-γ and IL-4, and none were positive for IL-2 mRNA 72 hours post-attachment during the primary infestation. During secondary and tertiary infestations, there were more IFN-γ and IL-2 mRNA positive cells, and although there was a peak of IL-4 mRNA positive cells during the second infestation, the number of IL-4 positive cells was never greater than that observed for IFN-γ and IL-2 (Mbow et al., 1994c). Keratinocytes, dermal dendritic cells, and mononuclear cells were positive for mRNA and at the protein level for both IL-1α and TNF-α during three successive infestations, with the intensity of TNF-α, but not IL-1α, increased during the secondary and tertiary infestations (Mbow et al., 1994b).

The cytokine (specifically IFN-γ) and cellular profiles reported by Mbow and colleagues (Mbow et al., 1994c) was correlated to the development of cutaneous delayed type hypersensitivity in infested mice, despite the lack of expression of acquired resistance to ticks (Mbow et al., 1994a). Unfortunately, in these studies, only one Th2 cytokine (IL-4) was assessed, and only at 72 hours post-attachment of ticks. However, more recent studies have examined the influence of tick feeding on the production of both Th1 and Th2 cytokines (Ferreira and Silva, 1999; Schoeler et al., 1999). Current

knowledge concerning the influence of tick feeding on host cytokine production will be discussed in the following section.

The extended period of time required for ticks to take a bloodmeal increases their exposure to host hemostatic and immune defenses (Wikel et al., 1996; Wikel, 1996a; 1999). To ensure successful feeding, ticks have developed countermeasures to host hemostatic and immune mechanisms.

Tick modulation of the host defenses: A brief discussion concerning the ability of ticks to modulate host hemostatic mechanisms will precede a more detailed overview of the host immune functions suppressed, or modified, by ticks. Homeostasis begins by the activation of circulating platelets by exposure to injury-associated agonists. Activated platelets form a plug, and degranulate to release adenosine diphosphate (ADP), serotonin, and thromboxane A_2 (Ribeiro, 1987b). The platelet plug is stabilized into a fibrin clot through the extrinsic pathway of coagulation, in response to tissue factor presented by activated platelets and non-vascular cells (Champagne and Valenzuela, 1996).

Tick saliva contains an array of pharmacologically active molecules that can act on a variety of host mechanisms inhibiting pain signals, blood clotting and coagulation, and increasing vasodialation (Ribeiro, 1987b; 1989; 1995). A metallo dipeptidyl carboxypeptidase in the saliva of *I. scapularis* acts as a kininase capable of inhibiting bradykinin, therefore inhibiting the pain signal during tick feeding (Ribeiro and Mather, 1998). Apyrase and second messenger activators present in the saliva of ticks function to block platelet aggregation. Tick salivary apyrase breaks down ADP, which has a role in platelet aggregation, into adenosine monophosphate (AMP) (Ribeiro, 1987b). In

A. americanum, apyrase is not present in the saliva, however, high concentrations of prostaglandins in the saliva function in platelet aggregation inhibition (Ribeiro et al., 1992). Inhibition of factors of VII and V of the intrinsic and extrinsic coagulation pathways by *D. andersoni* has been reported (Gorden and Allen, 1991). In addition, anti-factors IX and Xa have been identified in the salivary glands of *Orinthodoros moubata* and *Rhipicephalus appendiculatus*, respectively (Hellmann and Hawkins, 1967; Limo et al., 1991). Molecules that inhibit blood clotting and coagulation also have roles in vasodilation. The prostaglandins found in the saliva of *A. americanum* may act as a vasodilator and increase blood flow to mouthparts of feeding ticks (Ribeiro et al., 1992). Prostacyclin present in *I. scapularis* saliva can induce vasodilation and block platelet aggregation (Ribeiro et al., 1988).

Events in homeostasis and immune responses are interrelated (Wikel, 1996c). In addition to its ability to inhibit platelet aggregation, salivary apyrase may also prevent the accumulation of neutrophils and degranulation of mast cells, both of which are associated with the immune response to tick feeding (Wikel, 1996a). Tick-mediated immunosuppression is directed towards complement, NK cells, antibody production, antigen-presenting cells, and T cells (Ribeiro, 1987b; 1989; Wikel and Bergman, 1997; Wikel, 1996a; 1999).

Inactivation of complement is accomplished through inhibition of C3 hydrolysis, preventing formation of the anaphylatoxin C3a, and by preventing C3b and C5b from binding to sites where activation can occur (Ribeiro, 1987a; Ribeiro and Spielman, 1986). Furthermore, the inactivation of anaphylation inhibits smooth muscle contraction,

histamine release from mast cells and basophils, increased permeability of blood vessels, and C5a-mediated chemotaxis of neutrophils, eosinophils, basophils, and monocytes (Wikel, 1996c).

Suppression of NK cells, because of their ability to interact with antigen-presenting cells and subsets of $CD4^+$ T cells, might be important to feeding ticks (Wikel and Bergman, 1997; Wikel, 1999). An *in vitro* assay using salivary gland extracts (SGE) from *D. reticulatus* reported a decrease in the ability of NK cells to kill tumor targets (Kubes et al., 1994). Although the role of NK cells in the expression of acquired resistance has yet to be established, the regulation of cytokines produced by these cells is likely important to successful tick feeding (Wikel, 1999). Production of IFN-γ by NK cells, when stimulated by IL-2, enhances the ability of macrophage produced IL-12 to shift T cell differentiation towards a Th1 response (Trinchieri, 1995). Therefore, suppression of NK cell activity may reduce the Th1 response, and it is advantageous to the tick to suppress the pro-inflammatory response when feeding (Wikel and Bergman, 1997; Wikel, 1999).

A reduction in antibody responses has been identified in tick-infested laboratory animals. Guinea pigs infested with *D. andersoni* had a significantly reduced hemolytic plaque-forming cell IgM response to a thymic dependent antigen (Wikel, 1985). Another study, using rabbits infested with *R. appendiculatus*, also showed reduced antibody responses to bovine serum albumin (Fivaz, 1989).

Tick-induced host immunomodulation of macrophage and T lymphocytes are important countermeasures to acquired resistance to infestation (Wikel, 1996b; 1999).

Support for immunosuppression of lymphocyte functions has come from both infestation assays and the use of SGE and saliva in *in vitro* proliferation assays. Lymphocytes from guinea pigs infested twice with *D. andersoni* had reduced *in vitro* responsiveness to the T cell mitogen concanavalin A (Con A) during both infestations (Wikel, 1982). Similar results have been reported with a rabbit-*I. ricinus* model (Schorderet and Brossard, 1994). The ability of lymphocytes from tick-naive mice to proliferate in response to Con A, in the presence of SGE or saliva, has been examined for a number of ticks. *Dermacentor andersoni* SGE prepared daily from engorging females suppressed normal murine lymphocyte *in vitro* responsiveness to Con A (Ramachandra and Wikel, 1992). Saliva from both *I. scapularis* (Urioste et al., 1994) and *R. sanguineus* (Ferreira and Silva, 1998) also suppressed T lymphocyte responsiveness to mitogen *in vitro*.

Recently, the specific molecules present in salivary glands that have immunosuppressive characteristics are being identified (Bergman et al., 1995). A 36-kDa soluble protein in the salivary glands of *D. andersoni* has been found to suppress the Con A-induced proliferation of T lymphocytes (Bergman et al., 1998), furthermore, this protein has been molecularly cloned and is known to be secreted by adult female ticks (Bergman et al., 2000). The isolation and characterization of these immunosuppressant proteins expand the possibility for anti-tick vaccine development (Wikel, 1999).

Probably the most important effect of suppressed macrophage and lymphocyte function is the resultant modulation of cytokine production by these cells. Salivary gland extracts prepared daily from engorging female *D. andersoni* suppressed the elaboration of the macrophages cytokines IL-1 and TNF-α, as well as the T lymphocyte cytokines IL-2

and IFN-γ *in vitro* (Ramachandra and Wikel, 1992). The influence of infestation on cytokine production in the host was introduced in the previous section, presented here are the more recent studies, in which the effects of infestation on host T lymphocyte cytokine profiles are discussed.

Repeated infestation of hosts seems to shift the production of T lymphocyte cytokines toward a Th2 cell profile (Wikel, 1996b; 1999). The production of IL-4 and IFN-γ by axillary and brachial lymph node cells of BALB/c mice infested with *I. ricinus* nymphs were assessed nine days post-infestation (Ganapamo et al., 1995). During the first infestation levels of IL-4 were enhanced, and only low levels of IFN-γ were detected. Both IL-4 and IFN-γ were enhanced after the third infestation. Additionally, levels of IL-10 were found to be enhanced after both the first and third infestations, indicating a shift toward a Th2 response in infested mice (Ganapamo et al., 1996). C3H/HeJ mice repeatedly infested with *R. sanguineus* had significantly decreased levels of IL-2 and IFN-γ, while IL-4 and IL-10 were significantly enhanced, after the first and fourth infestations (Ferreira and Silva, 1999). One infestation of C3H/HeJ mice with pathogen-free nymphal *I. ricinus* ticks also showed a suppression of IL-2 (-45%) and IFN-γ (-74%) beginning six and two days, respectively, post-infestation, while IL-4 and IL-10 were upregulated during infestation periods, with the levels of IL-4 and IL-10 remaining elevated 12 days after initial infestation (Zeidner et al., 1997). Again, providing evidence for selective polarization of cytokine production toward a Th2 profile.

A more detailed study examining the influence of *I. scapularis* on both BALB/c and C3H/HeN mice through four successive infestations confirmed the polarization of

cytokine production toward a Th2 profile (Schoeler et al., 1999). In two independent trials, significant increases in IL-4 and IL-10 were observed during infestations for both BALB/c and C3H/HeN mice. Significant suppression of IL-2 and IFN-γ was observed in C3H/HeN mice, and to a lesser extent in BALB/c mice during each infestation. Throughout this study, and the other studies discussed, acquired resistance to tick feeding did not develop in hosts, suggesting the importance of Th1-mediated immune responses in the expression of acquired resistance (Wikel, 1999).

Information concerning tick modulation of the host immune response has been reported, providing clues as to which immune mechanisms are required for the expression of acquired resistance (Wikel, 1996a; 1999). However, the ability of ticks to modulate the molecules responsible for cellular trafficking remain relatively unexplored. Additionally, the T lymphocyte cytokine profile in tick-resistant hosts may differ from those reported in hosts that do not express acquired resistance. Valuable information, concerning the expression of acquired resistance, can be gathered by examination of tick influence on both adhesion molecule expression and cytokine production in tick-resistant hosts.

Current study

The influence of tick salivary gland derived molecules on the expression of adhesion molecules by endothelial cells and lymphocytes is unknown. In light of how ticks modulate various aspects of the host immune response, the effect of tick salivary gland-derived molecules on cellular adhesion molecule expression was investigated. Utilizing *in vitro* techniques, the effect of molecules present on salivary gland extract can

be assessed. Furthermore, by using saliva from partially engorged ticks, it can be determined that molecules of the salivary gland which are secreted during feeding induced changes in an expression.

To complement *in vitro* research using salivary gland preparations, the use of infestation assays can provide insight into the actual effect of tick feeding on adhesion molecule expression by host lymphocytes. Down regulation of adhesion molecule expression could play a key role in cellular migration to tick attachment sites, and this regulation of expression is likely related to other lymphocyte functions. As discussed earlier, adhesion molecule expression and subsequent lymphocyte trafficking are dependent on cytokine signals from cells involved in the inflammatory response. The type of immune response generated is dependent on the specific cytokines produced by effector cells, specifically T cells. Proliferation and differentiation of T cells during tick feeding may be important in developing and effective immune response, leading to the formation of acquired resistance. By measuring T cell responses of hosts that express acquired resistance during repeat infestations, it might be possible to identify mechanisms that are involved in the development of acquired resistance.

Hypotheses and specific aims

This research is based on the hypothesis that exposure of *in vitro* cultured murine endothelial cells and BALB\c lymphocytes to *D. andersoni* SGE/saliva, down regulates expression of cell surface adhesion molecules. Specific aims include: 1) determining the effects of SGE/saliva on the constitutive and stimulus-induced expression of the endothelial adhesion molecules E-selectin, VCAM-1, and ICAM-1; and, 2) determining

the effects of SGE/saliva on the constitutive and stimulated expression of the lymphocyte integrins LFA-1 and VLA-4. The second hypothesis for this research is that infestation of mice with *D. andersoni* nymphs will down regulate expression of lymphocyte adhesion molecules, as well as, modulate related T cell functions, including mitogen and antigen-driven lymphocyte responses and cytokine production by infested mice. Specific aims include: 1) determining the effects of repeated infestation on the constitutive and stimulated expression of the lymphocyte integrins LFA-1 and VLA-4; 2) determining the effects of repeated infestation on the mitogen responsiveness of lymphocytes; 3) determining the effects of repeated infestation on the antigen recognition by lymphocytes; and, 4) determining the effects of repeated infestation on T lymphocyte cytokine production.

CHAPTER II

MATERIALS AND METHODS

Experiments were designed to examine the ability of SGE or saliva collected from *D. andersoni* to modulate the cell surface expression *in vitro* of adhesion molecules by murine endothelial cells and lymphocytes. The constitutive and stimulus-induced expression of E-selectin, VCAM-1, and ICAM-1 by endothelial cells were measured at four time points over a 24 hour period, by a whole-cell ELISA developed in this study. Cells were pre-treated with either SGE or saliva diluted in 0.1 M phosphate buffered saline (PBS, pH 7.2, Appendix I, Section 1), cultured in the presence or absence of a stimulus, and adhesion molecule expression was measured. Endothelial cells pre-treated with PBS alone served as a control for the tick-derived salivary gland material.

In addition, experiments were performed to determine the effects of repeated exposure of BALB/c mice with *D. andersoni* nymphs on the expression of adhesion molecules; mitogen-induced proliferation; tick salivary gland-derived antigen recognition; and, production of cytokines. Splenocytes were collected from infested and control mice for use in a number of assays. Expression of the lymphocyte adhesion molecules LFA-1 and VLA-4 was measured using whole-cell ELISA. *In vitro* splenocyte proliferation assays were conducted to assess immunosuppression of infested animals, as well as the ability of splenocytes to specifically proliferate in response to tick salivary gland antigens. Production of T lymphocyte cytokines was measured using antigen-capture ELISA and recombinant cytokine standards.

Tick Colony

Dermacentor andersoni adults and nymphs used in this project were obtained from the colony of the Department of Entomology and Plant Pathology, Oklahoma State University. At this facility, larvae and nymphs are fed on rabbits caged over water. After engorgement, ticks which detach and fall into the water are collected daily. Sheep serve as hosts for adult ticks, which are enclosed in tubular medical stockinette cells on the shaved back of the host. All tick life stages are maintained in a 14:10 (L:D) photoperiod at 25°C over a super-saturated solution of potassium nitrate (relative humidity = 93%), according to procedures described by Patrick and Hair (1975).

Tick Salivary Gland Extract and Saliva Preparation

Salivary gland collection and extract preparation were performed according to procedures described by Ramachandra and Wikel (1992). Unmated adult female ticks were fed for four days on previously unexposed sheep. Upon removal from the host, ticks were surface-sterilized by sequential washing in sterile distilled H_2O, 0.5% sodium hypochlorite, 1.0% benzalkonium chloride, 70% ethanol, sterile double-distilled H_2O, and sterile distilled H_2O containing 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Grand Island, NY). Ticks were cut into anterior and posterior halves and placed into the bottom half of a plastic petri dish, containing PBS. Salivary glands were removed and placed into 50 μl of chilled PBS in a 1.5 ml centrifuge tube held on ice. Fifty pairs of salivary glands were collected per tube, and washed three times in PBS with centrifugation at 150 x g for ten minutes at room temperature (RT; 22°C). Samples were immediately frozen and stored at -20°C until used.

Tick salivary glands were frozen and thawed once prior to three one minute cycles of sonication at 55,000 cycles/s, during which the sample tubes were chilled in an ice bath. Salivary gland extracts were sterilized by passage through a 0.22 μm filter (Millipore Corp., Bedford, MA). Protein concentration of the salivary gland sonicate was determined by the bicinchoninic acid (BCA) microassay (Pierce Chemical Company, Rockford, IL) described by (Smith et al., 1985). Bovine serum albumin (BSA; 2 mg/ml, in a 0.9 % NaCl solution, Pierce), used as the standard for the assay, was diluted in PBS to concentrations of 50, 100, and 150 μg/ml. Sterile salivary gland sonicate was serially diluted in PBS at dilutions of 1:20 to 1:640.

Tick saliva was collected from four to six day fed *D. andersoni* females. Ticks were surface sterilized as described above, and induced to salivate by injection of dopamine (1.0 mg/ml in 150 mM NaCl, containing 2 mM $CaCl_2$ and 3% DMSO) into the hemocoel. Injections (10 μl) were given 3 to 5 times at 10 minute intervals. Saliva was collected by placing a 10 μl capillary tube over the mouthparts. Collected saliva was placed into a 1.5 ml centrifuge tube, containing 50 μl of PBS, and held on ice. Saliva was immediately dialyzed for 48 hours against PBS, using a 3.5 kdal molecular weight cut off dialysis cassette (Pierce) at 4°C. After dialysis, saliva was sterilized by passage through a 0.22 μm filter (Millipore). Protein concentration was determined by BCA microassay (Pierce). Saliva was aliquoted and stored at -20°C until used.

Endothelial Cell Culture

A murine endothelial cell line (bEnd3), derived from brain capillary endothelial cells (Montesano *et al.*, 1990), was used in all experiments. This cell line was a gift from

Dr. C. H. Lee (Naval Medical Research Institute, Bethesda, MD). The cell line was maintained in growth media consisting of 90% high glucose (4.5 grams/ liter) Dulbecco's Modified Eagle Medium (DMEM; Appendix I, Section 1; Gibco BRL, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT) along with 100 units/ml penicillin and 100μg/ml streptomycin and cultured at 37°C in a humidified 5% CO_2 atmosphere.

Upon receiving the endothelial cell line, frozen cells were rapidly thawed by holding the vial in a 37°C water bath. The initial cell population was transferred to a 15 ml conical tissue culture tube, and washed two times in DMEM containing 100 units/ml penicillin and 100μg/ml streptomycin by centrifugation (150 x g for nine minutes). Cells were suspended in 2 ml of growth media and counted.

A cell suspension for counting was prepared in a 96-well flat-bottomed tissue culture plate (Corning) by placing 10 μl of cell suspension into 90 μl of Turk's solution [0.01 g gentian violet, 3 ml glacial acidic acid, and 97 ml distilled water], prior to mixing by gentle pipetting. The mixture was transferred to the hematocytometer and cells were counted at 100 x magnification. Opposing diagonal squares were counted and a mean was calculated. Cells per ml were calculated by the formula: Mean cell count x dilution factor (1:10) x 10^4. Cell suspension was then adjusted to the desired concentration.

Endothelial cells were seeded at a concentration of 5 x 10^6 cells/ml and maintained in T-75 tissue culture flasks (Corning). Throughout this study, uniform populations of low passage (passage $\leq$ 10) cells were maintained by development of a master seed of cells. A master seed was created by splitting the initial cell culture

(passage 3) twice and freezing cells at the fifth passage at a concentration of 5 x 10^6 cells/ml in freeze media (DMEM with 10% DMSO) in 1 ml aliquots in cyrovials (Nalgene). Cryovials were stored overnight at -80°C, then transferred to liquid nitrogen for long term storage. After re-culturing from the master seed, cell populations were passed no more than five times for use in endothelial cell adhesion molecule assays.

Endothelial Cell Assay

Endothelial cells were removed from culture flasks by incubation with trypsin/EDTA solution for three minutes at 37°C in a humidified 5% CO_2 atmosphere. After which time, an equal volume of DMEM was added to the flask. The new media and cell suspension were mixed by gentle pipetting. The cell suspension was transferred to a 25 ml conical tissue culture tube. Cells were isolated by centrifugation (150 x g for nine minutes) and resuspended in 2 ml of growth medium. Cells were counted as described above, and adjusted to a concentration of 1.0 x 10^5 cells/ml. One hundred μl of the cell suspension was seeded into each well of a 96-well flat-bottomed tissue culture plate. Cells were cultured (37°C in a humidified 5% CO_2 atmosphere) for approximately two days, at which time a confluent monolayer of cells had formed.

The effects of tick salivary gland derived molecules on the constitutive level of expression of adhesion molecules was determined by incubating cells with 20 μl of growth media, PBS, or a range of concentrations of tick SGE or saliva (1.0 to 10.0 μg/ml; 0.1 to 1.0 μg tick protein diluted in 20 μl of growth media or PBS) for up to 24 hours. To determine the effects of tick salivary gland derived molecules on the stimulus-induced expression of adhesion molecules, specific wells were first incubated with 20 μl of

growth media, PBS, tick SGE, or saliva, at the same concentrations described above, for 2 hours. These cells were then incubated with either *Escherichia coli* lipopolysaccharide (LPS, serotype O55:B5, 1.0 μg/ml; 0.1 μg in 20 μl of growth media) or murine recombinant TNF-α (mr TNF-α, 10.0 ng/ml; 1.0 ng in 20 μl of growth media) for up to an additional 24 hours. Monolayers not cultured in the presence of tick protein, but cultured with either LPS or mr TNF-α, served as positive controls for stimulated cells. The total volume of each well was 140 μl. After the second incubation period, monolayers were used for either cell viability assays or whole-cell ELISA to measure expression of adhesion molecules.

Measurement of Endothelial Cell Viability

The viability of endothelial cells was monitored using a colorimetric assay, with the tetrzolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfopheny)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES) (Promega, Madison, WI). The MTS tetrazolium compound is bioreduced by metabolically active cells into a colored formazan product that is soluble in tissue culture medium. Cell monolayers were incubated with 28 μl of CellTiter96® Aqueous One Solution Reagent (Promega) for one hour at 37°C in a humidified 5% CO_2 atmosphere. Color intensity was measured by absorbance at 490 nm using a microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT). Background absorbance was determined by measuring wells containing only culture medium.

Measurement of Endothelial Cell Expression of Adhesion Molecules *in vitro* using Whole-cell ELISA

Endothelial cell ELISAs were modified from the procedure described by Boggemeyer et al. (1994). Recipes for buffers and reagents used are provided in Appendix I (Section 2). The levels of expression of adhesion molecules were determined using monoclonal antibodies (Mabs): rat [IgG]-anti-mouse CD106 (VCAM-1; PharMingen, San Diego, CA), rat [IgG]-anti-mouse CD62E (E-selectin; PharMingen), and rat [IgG]-anti-mouse CD54 (ICAM-1; Cedarlane, Ontario, Canada). These Mabs have been shown to block functional interactions of these adhesion molecules with their respective ligands E-selectin (Ramos et al., 1997), VCAM-1 (Kumar et al., 1994), and ICAM-1 (Kurachi et al., 1993). Secondary antibody was biotin-SP-conjugated goat-anti-rat IgG, heavy and light chain specific (Jackson ImmunoResearch Laboratories, West Grove, PA). Streptavidin-horseradish peroxidase (HRPO) conjugate (PharMingen), o-phenylenediamine-dihydrochloride (OPD; Sigma Chemical Company, St. Louis, MO), and H_2O_2 were used to detect immobilized specific antibodies.

Cell monolayers were washed between steps by filling each well with ice-cold PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/Ca^{2+}/Mg^{2+}), and allowing it stand at RT for one minute, prior to drying the wells by tapping the top of the wells against paper towels or other absorbent material.

Cell monolayers were washed three times and incubated with 100 μl of the first antibody (E-selectin and VCAM-1, 0.5 mg/ml stock diluted 1:500 in 1% BSA/PBS; ICAM-1, 0.2 mg/ml stock diluted 1:50 in 1% BSA/PBS) for one hour on ice. Cell

monolayers were then washed three times with PBS/Ca^{2+}/Mg^{2+} and fixed by adding 100 μl of 4% paraformaldehyde/PBS to the wells, and incubating plates for 15 minutes on ice. After three washes (PBS/Ca^{2+}/Mg^{2+}), cell monolayers were incubated with 100 μl of the secondary antibody (1.5 mg/ml stock diluted 1:5000 in 1% BSA/PBS) for 30 minutes at RT. After three more washes (PBS/Ca^{2+}/Mg^{2+}), cell monolayers were incubated with 100 μl of streptavidin-HRPO conjugate (diluted 1:1000) at RT for 30 minutes. Cell monolayers were then washed eight times (PBS/Ca^{2+}/Mg^{2+}), and incubated with 100 μl of o-phenylenediamine-dihydrochloride (OPD; 300μg/ml, 50 mM K_2HPO_4, 25 mM citric acid, pH 6.0) with 0.006% H_2O_2 for 30 minutes at RT. The reaction was stopped by adding 50 μl of HCl to obtain a 1 mM final concentration in the wells. Absorbance was measured at 490 nm, using a microplate spectrophotometer (Bio-Tek Instruments Inc.). Absorbance of test wells were blanked against the optical densities obtained from cells incubated with secondary antibody, streptavidin-HRPO, and substrate alone.

Experimental Animals

Mice. Female BALB/c mice, 8 to 12 weeks old, were obtained from Jackson Laboratories, Bar Harbor, ME. Mice were housed in the Laboratory Animal Resource Facility of Oklahoma State University at RT and fed a commercial diet and water *ad libitum.*

Splenocyte Recovery

Splenocytes were prepared according to procedures described by Ramachandra and Wikel (1992). Wash media used for splenocyte preparation consisted of RPMI 1640 [Appendix I, Section I] (Life Technologies Inc., Gaithersburg, MD), containing 200

units/ml penicillin, and 200 μg/ml streptomycin. Growth media consisting of RPMI 1640 with 100 units/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated FBS (HyClone) was used for splenocyte culture. Spleens were dissociated between the frosted ends of sterile microscope slides (Fisher Scientific, Pittsburgh, PA) bathed in wash media. Cell suspension was then passed through a nylon filter and washed twice by centrifugation (150 x g for 10 minutes at 4° C) in wash media. Cells were then suspended at a concentration of 5 x 10^6 cells/ml in growth media.

Measurement of Lymphocyte Expression of Adhesion Molecules *in vitro* using Whole-cell ELISA

Splenocytes collected from tick-naive BALB/c mice were seeded at a concentration of 5.0 x 10^6 cells/ml in a 24-well flat bottomed tissue culture plate. Each well, containing 1 ml of cell suspension was incubated with 20 μl of growth medium, PBS alone, tick SGE or saliva (0.1 or 1.0 μg in 20 μl of sterile PBS) for 2 hour at 37°C in a humidified 5% CO_2 atmosphere. These cells were then incubated for 46 to 48 hours with an additional 25 μl growth medium or Con A (2.5 μg in 25 μl). After the second incubation, cells were removed from the 24-well plate by gentle pipetting, counted as previously described, and seeded in triplicate at a concentration of 2.5 x 10^5 cells per well into a 96-well U-bottomed plate (Corning), for use in the whole-cell ELISA.

A modification of the endothelial cell ELISA was developed to measure expression of the lymphocyte integrins, LFA-1 and VLA-4. Because splenocytes were non-adherent to the bottom of the wells, a technique to wash the cells by centrifugation was developed. The wash buffer and other reagents used in this ELISA were the same as

those described for the endothelial cell ELISA (Appendix I, Section 2). Monoclonal antibodies used in this procedure, rat [IgG]-anti-mouse CD11a (LFA-1; PharMingen) and rat [IgG]-anti-mouse CD49d (VLA-4; PharMingen), have been shown to block functional interactions of these adhesion molecules with their respective ligands, ICAM-1 (Wuthrich, 1991) and VCAM-1 (Ferguson and Kupper, 1993). The detection antibody was biotin-SP-conjugated goat-anti-rat IgG, heavy and light chain (Jackson ImmunoResearch). The enzyme used was streptavidin-HRPO conjugate (PharMingen), and OPD with H_2O_2 was the substrate.

During this ELISA, splenocytes were washed between steps by centrifugation. One hundred μl of ice-cold PBS/Ca^{2+}/Mg^{2+} were added to each well. Plates were centrifuged at 450 x g for three minutes at 4° C, after which time the splenocytes were located along the bottom of each well. Excess supernatant was removed. After addition of wash buffer, or appropriate ELISA reagent, splenocytes were resuspended in the wells by gentle agitation of the bottom of the wells.

After cells were removed from the 24-well flat-bottomed tissue culture plate and seeded into the 96-well U-bottomed plate, splenocytes were washed three times with PBS/Ca^{2+}/Mg^{2+}, and incubated with the first antibody (LFA-1 and VLA-4, 0.5 mg/ml stock diluted 1:500 in 1% BSA/PBS) for 1 hour on ice. Cells were then washed three times with PBS/Ca^{2+}/Mg^{2+} and fixed with 4% paraformaldehyde in PBS for 15 minutes on ice. After three additional washes (PBS/Ca^{2+}/Mg^{2+}), cells were incubated with the secondary antibody (1.5 mg/ml stock diluted 1:5000 in 1% BSA/PBS) for 30 minutes at RT. After three washes (PBS/Ca^{2+}/Mg^{2+}), cells were incubated with streptavidin-HRPO

conjugate (diluted 1:1000) at RT for 30 minutes. Cells were then washed eight times (PBS/Ca^{2+}/Mg^{2+}), and incubated with OPD (300μg/ml, 50 mM K_2HPO_4, 25 mM citric acid, pH 6.0 with 0.006% H_2O_2) for 30 minutes at RT. The reaction was stopped by adding HCl to obtain a final concentration of 1 mM. Absorbance was measured at 490 nm by use of a microplate spectrophotometer (Bio-Tek Instruments Inc.). Optical density of each test well was blanked against the OD obtained for cells treated with only secondary antibody, streptavidin-HRPO, and substrate.

Tick Infestation

Mice used for infestation assays were divided into two groups, and each of those groups was further subdivided into infested and uninfested control mice. The first group of infested mice were exposed once to ticks, while the second group of infested mice were exposed twice. Infestation was done according to procedures described by (Schoeler et al., 1999). Both infested and control mice were harnessed with a 1.5 ml microcentrifuge tube, cut in half, that was secured to the shaved back of the mouse with a 4/1 (w/w) mixture of calophonium (rosin) and beeswax. For control and infested mice of the twice infested group, a new skin site for capsule attachment was used for each infestation. Each infested mouse received 10 *D. andersoni* nymphs for each exposure. Nymphs were allowed to feed until fully engorged or for a maximum of seven days. A 14 day tick-free period was maintained between infestations.

Tick Biology Parameters

The tick biology parameters of weight and viability were recorded for ticks collected after each infestation. Tick weight was recorded the day ticks detached or were

removed from mice. Tick viability was determined as the percentage of recovered nymphs that successfully molted to adults.

Measurement of Lymphocyte Expression of Adhesion Molecules by Cells Derived from Infested Mice

The effects of tick infestation on the expression of adhesion molecules by lymphocytes was assessed. Splenic lymphocytes of infested and control mice were seeded at a concentration of 5.0 x 10^6 cells/ml in a 24-well flat-bottomed tissue culture plate. Each well, containing 1 ml of cell suspension, was incubated for 48 h with an additional 25 μl growth medium or Con A (2.5 μg in 25 μl of growth medium). Cells were removed from the 24-well plate by gentle pipetting, counted as previously described, and seeded in triplicate at 2.5 x 10^5 cells per well into a 96-well U-bottomed plate. The whole-cell ELISA used to measure expression of LFA-1 and VLA-4 was the same as that previously described (see *Measurement of lymphocyte expression of adhesion molecules in vitro using whole-cell ELISA*).

Mitogen-Induced *in vitro* Splenocyte Proliferation

To evaluate the effect of infestation on Con A-induced blastogenesis, the lymphocyte proliferation assay described by Ramachandra and Wikel (1992) was used. A range of concentrations of Con A (0.5 to 2.0 μg in 100 μl of growth media; RPMI 1640 with 100 units/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated FBS) were added to the appropriate wells of a 96-well flat-bottomed tissue culture plate. Splenocyte suspensions consisting of 100 μl of 5.0 x 10^6 cells/ml from either control or infested mice were added, in triplicate, to the wells of the 96-well flat-bottomed tissue culture plate, and

cells were cultured for 54 hours at 37°C in a humidified 5% CO_2 atmosphere. One microcurie of methyl-^{3}H-thymidine with a specific activity of 248 Gbq/mmol (NEN Life Science Products, Boston, MA) was added to each well and plates were cultured for an additional 17 hours. Incorporation of radioisotope was determined by collecting cellular nucleic acids onto glass fiber filters by use of an automated cell harvester (Brandel Laboratories, Gaithersburg, MD) and measuring activity by use of a liquid scintillation counter (Model LS6000SC, Beckman Instruments Inc.). Baseline incorporation of radioisotope by splenocytes was determined by culturing cells in growth media lacking Con A. Mean counts per minute (CPM) ± S.E.M. were calculated for each treatment. A stimulation index (SI) was calculated for each treatment and mouse, by dividing the treated well (Con A) by the control well (media without Con A). Stimulation indices were used to normalize lymphocyte *in vitro* responses based on radioisotope incorporation by unstimulated cells. Comparisons were made between control and infested mice for each concentration of Con A and between the two infestations.

Antigen-Induced *in vitro* Splenocyte Proliferation

Lymphocyte proliferation in response to tick salivary gland extract or saliva immunogens were assessed by the methods of Ramachandra and Wikel (1992). A range of concentrations of SGE or saliva (SGE and saliva; 0.1 to 1.0 μg in 20 μl of sterile PBS + 80 μl of growth media) were added to the appropriate wells of a 96-well flat-bottomed tissue culture plate. Splenocyte suspensions of 100 μl of 5.0 x 10^6 cells/ml from either control or infested mice were added, in triplicate and cells were cultured for 54 hours at 37°C in a humidified 5% CO_2 atmosphere. One microcurie of methyl-^{3}H-thymidine with

a specific activity of 248 Gbq/mmol was added to each well and the plates were cultured for an additional 17 hours. Incorporation of radioisotope was determined by collecting cellular nucleic acids onto glass fiber filters by use of an automated cell harvester and measuring activity by use of a liquid scintillation spectrophotometer. Baseline incorporation of radioisotope by splenocytes was determined by culturing cells in growth media lacking SGE or saliva. Mean CPM ± S.E.M. were calculated for each treatment. An SI was calculated for each treatment and mouse, by dividing the treated well (tick salivary gland extract or saliva) by the control well (PBS without tick immunogens). Stimulation indices were used to normalize lymphocyte *in vitro* responses based on radioisotope incorporation by unstimulated cells. Comparisons were made between control and infested mice for each infestation.

T lymphocyte Production of Cytokines

Cytokine induction and measurement were performed according to procedures described by Schoeler et al. (1999). Splenocytes from control and infested mice used in infestation assays were suspended at a concentration of 2.5 x 10^6 cells/ml in growth media and seeded at 1 ml/well in a 24-well flat-bottomed tissue culture plate. Each well was cultured with 5.0 μg/ml of Con A, added to the culture in 20 μl, prior to incubation for 48 h at 37°C in a humidified 5% CO_2 atmosphere.

Concentrations of IL-2, IL-4, IL-10, and IFN-γ were quantitated by antigen capture ELISA. Opt-EIA ELISA kits for each cytokine were purchased from PharMingen, Inc. (San Diego, CA), and reagents and protocols provided by the

manufacturer were used. Formulations of buffers and reagents used in these ELISAs are provided in the Appendix I (Section 2).

One hundred μl of purified anti-cytokine capture antibody, diluted in a binding solution (0.1 M Na_2HPO_4, pH 9.5), were added to the wells of an enhanced protein binding ELISA plate (NalgeNunc, Rochester, NY). The plate was sealed and incubated overnight at 4°C. The plate was washed three times with wash buffer (PBS with 0.05% Tween-20) prior to blocking with 200 μl/well of assay diluent (PBS with 10% heat-inactivated fetal bovine serum, pH 7.0) and RT for one hour. After blocking, the plates were washed three times (PBS/Tween).

For IL-2, IL-4, and IL-10 assays, 100 μl of recombinant cytokine standard or culture supernatant were added, in triplicate, to appropriate wells. Plates were sealed and incubated for 2 hours at RT. After five washes (PBS/Tween), 100 μl of working detector (biotinylated detection antibody and streptavidin-HRPO conjugate diluted in assay diluent) were added to appropriate plates and incubated for one hour at RT. IL-2, IL-4, and IL-10 plates were then washed ten times (PBS/Tween) and 100 μl of substrate solution (tetramethylbenzidine and H_2O_2) were added to the wells and plates were incubated at RT for 30 minutes in the dark. The reaction was stopped with the addition of 50 μl of 2 N H_2SO_4 to each well, and absorbance was measured at 450 nm, using a microplate spectrophotometer (Bio-Tek Instruments Inc.). Optical densities of test wells were blanked against the OD obtained from wells containing all reagents, with the exception that assay diluent replaced cytokine standard or sample.

For the IFN-γ assay, after blocking the wells, 50 μl of recombinant cytokine standard or culture supernatant and 50 μl of diluted biotinylated detection antibody were added, in triplicate, to appropriate wells. After five washes (PBS/Tween), 100 μl of diluted streptavidin-HRPO conjugate diluted in assay diluent were added to appropriate wells and incubated for 30 minutes at RT. The plate was then washed ten times (PBS/Tween) and 100 μl of substrate solution (tetramethylbenzidine and H_2O_2) were added to the wells and the plate was incubated at RT for 30 minutes in the dark. The reaction was stopped with the addition of 50 μl of 2 N H_2SO_4 to each well, and absorbance was measured at 450 nm, using a microplate spectrophotometer (Bio-Tek Instruments Inc.). Optical densities of test wells were blanked against the mean ODs obtained from wells containing all reagents, with the exception that assay diluent replaced cytokine standard or sample.

Mean ODs for each concentration of recombinant cytokine standard (15.6 to 2000 pg/ml) were used to create a standard curve. For each assay, the concentration and mean OD for each standard were log-transformed and a linear regression was constructed to produce a regression equation. Cytokine concentrations in supernatants from control and infested mice were determined, using the regression equation calculated for each assay. Concentration of cytokines in supernatants were multiplied by the appropriate dilution factor to determine concentration per milliliter for each sample.

Experimental Design

To test the hypothesis that molecules present in tick salivary glands are capable of down-regulating the constitutive and stimulus-induced level of expression of endothelial

adhesion molecules *in vitro*, endothelial cell monolayers were cultured in the presence or absence of varying concentrations of tick protein, and expression of E-selectin, VCAM-1, and ICAM-1 was assessed by whole-cell ELISA. Peak stimulus-induced expression of endothelial adhesion molecules was reported to occur between zero and 24 hours of stimulation (Boggemeyer et al., 1994). Therefore, endothelial cell whole-cell ELISAs were carried out at 4, 8, 16, and 24 hour time points. To determine the effects of tick protein on adhesion molecule expression, monolayers cultured in the presence of SGE or saliva were compared to monolayers cultured in media alone. To assess the ability of molecules present in tick salivary glands to down regulate stimulus-induced expression of adhesion molecules, monolayers were cultured with varying concentration of either SGE or saliva for 2 hours, before the addition of a stimulus. Comparisons were made between wells that were pre-treated with SGE or saliva, before the addition of a stimulus, and wells that were cultured with only the stimulus. Because the tick protein was diluted in PBS, monolayers cultured with PBS alone (without tick protein) served as a control for the tick protein.

To determine the effects of tick protein on the *in vitro* expression of lymphocyte integrins, LFA-1 and VLA-4, another assay was designed. For this assay, splenocytes were recovered from tick-naive BALB/c mice and cultured for 48 hours with varying concentrations of tick protein, in the presence or absence of the T cell mitogen Con A. Cells were removed from the 24-well plate, counted, and seeded at a concentration of 2.5 x 10^5 cells/well into a 96-well plate. Whole-cell ELISAs were used to measure the level of expression of LFA-1 and VLA-4. Modulation of the constitutive levels of expression

of LFA-1 and VLA-4 was determined by comparing cells that had been cultured with tick SGE or saliva to cells that had been cultured in media alone. Salivary gland extract-induced suppression of Con A-induced lymphocyte responses has been previously reported (Ramachandra and Wikel, 1992). To examine the ability of SGE and saliva to suppress Con A induced expression of lymphocyte adhesion molecules, lymphocytes were pre-treated with varying concentrations of SGE or saliva prior to the addition of Con A to the culture. Whole-cell ELISAs were used to measure the level of expression of LFA-1 and VLA-4 after 48 hours of culture with Con A, and comparisons were made between cells pre-treated with SGE or saliva and cells cultured with media alone, in the presence of Con A. Similar to the endothelial cell assay, cells cultured with PBS alone (without tick protein) served as a control for tick protein for both constitutive and Con A-induced expression.

In order to assess the effects of tick SGE and saliva on the expression of adhesion molecules by both murine endothelial cells and lymphocytes *in vitro*, a minimum of three independent whole-cell ELISAs were carried out for each adhesion molecule. For each ELISA, each treatment was assessed in triplicate. A mean optical density ± S.E.M. was calculated for each adhesion molecule by combining the replicates of each experiment.

To address the second specific aim and test the hypothesis that tick feeding can modulate host lymphocyte responses, the effects of tick feeding on a number of lymphocyte functions were assessed. Three independent infestation trials were completed, and a total of 52 BALB/c mice were used in infestation assays. Data from the three infestations were combined and means are reported for a sample size of 13 control

mice and 13 tick exposed mice per infestation. After each infestation, splenocytes were immediately isolated from control and infested mice, and T lymphocyte responses including, *in vitro* proliferation, cytokine production, and lymphocyte expression of adhesion molecules were determined.

For each mouse, the effects of tick feeding on lymphocyte adhesion molecule expression was measured. Splenocytes from control and infested mice were cultured in the presence or absence of Con A for 48 hours. The ability of lymphocytes to respond to Con A, in terms of adhesion molecule expression, was measured using whole-cell ELISA. Comparisons were made between infested mice and control mice for each group. The constitutive level of expression of lymphocyte adhesion molecules was also examined for both groups of mice.

After each infestation, mitogen driven *in vitro* splenocyte proliferation assays were conducted to assess immunosuppression in infested animals. Splenocytes recovered from control and infested mice were cultured with varying concentrations of Con A for 54 hours, and the ability of these cells to proliferate in response to the mitogen was measured by determining the amount of radioisotope incorporated into new cells during a 17 hour incubation period following the addition of Con A. A stimulation index was calculated for each concentration of Con A. The magnitude of suppression was calculated by comparing stimulation indices of infested and uninfested control mice.

The ability of splenocytes to proliferate in response to tick salivary gland antigens was assessed by *in vitro* splenocyte proliferation assays. After each infestation, splenocytes recovered from control and infested mice were cultured with a range of

concentrations of SGE or saliva collected from adult *D. andersoni* for 54 hours prior to addition of methyl-^{3}H-thymidine and determination of radioisotope incorporation. A stimulation index was calculated for each concentration of SGE or saliva, and comparisons in stimulation indices were made between control and infested mice. Increased recognition of tick immunogens by tick-exposed mice was evident if stimulation indices of infested mice were greater than those observed for control mice.

The T lymphocyte cytokine profile, Th 1 or Th 2, was assessed for mice after one and two infestations. After each infestation, splenocytes were cultured with Con A for 48 hours, and supernatant of each mouse was collected. Cytokine-specific antigen-capture ELISAs were performed to measurement the amount of T lymphocyte cytokines in the supernatant. A regression equation was calculated for each ELISA using recombinant cytokine standards. Concentrations of cytokines in supernatants from infested and control mice were determined by using the regression equation calculated for the standards. Comparisons were made between infested and control mice after each infestation. To assess the profile of cytokines produced, and determine if polarization of cytokine production occurred, comparisons were made between the amounts of Th 1 and Th 2 cytokines produced by lymphocytes of infested versus control mice for the first and second infestations.

Data Analysis

Means of absorbance, tick biology parameters, stimulation indexes, and concentration of cytokines were evaluated for statistical significance by a 1-way ANOVA

with Fisher's least significant difference mean separation test (Steel et al., 1997). A *P*-value ≤ 0.05 was considered significant.

CHAPTER III

RESULTS

Endothelial Cell Viability

Cell viability of the bEnd3 cells incubated with SGE or PBS, in the presence of *E. coli* LPS, was not significantly different from cells incubated with media alone for 4 to 16 hours (without LPS) or 4 to 24 hours (with LPS) (Table 1). At 24 hours, unstimulated endothelial cells cultured with 1.0 μg SGE or PBS alone resulted in significant increases in cellular growth, compared to cells cultured with media alone.

Endothelial Cell Expression of Adhesion Molecules *in vitro*

E-selectin: The constitutive levels of expression of E-selectin (mean OD ± S.E.M.) by bEnd3 cells cultured with media, SGE, saliva, or PBS are shown in Table 2. The baseline constitutive level of expression of E-selectin from 4 to 24 hours averaged 0.168 ± 0.006. This constitutive level of expression was decreased in a dose-dependent manner when cells were incubated with SGE (0.1 μg = -16.6%; 0.5 μg = -18.3; 1.0 μg = -21.7%) or saliva (0.1 μg = -4.6%; 0.5 μg = -11.4; 1.0 μg = -14.3%) for 4 hours. At the highest concentration of SGE, 1.0 μg, the level of expression (0.137 ± 0.008) was significantly lower than that observed for cells incubated with media alone (OD = 0.175 ± 0.005). Neither SGE or saliva significantly modulated the constitutive levels of expression at 8 or 16 hours. However, again at 24 hours, both SGE and saliva significantly reduced the constitutive level of expression of E-selectin (0.173 ± 0.010). Similar to the suppression observed at 4 hours, decreases in expression by cells cultured with SGE were in a dose-

Table 1. Cell viability of bEnd3 endothelial cells incubated with media, SGE, or PBS, in the presence or absence of *E. coli* LPS for 4 to 24 hours.

	Incubation period (hours)			
Treatment	**4**	**8**	**16**	**24**
Media	0.760 ± 0.023 a	1.014 ± 0.019 a	1.011 ± 0.040 a	1.024 ± 0.046 d
Media + 1.0 μg SGE	0.767 ± 0.021 a	1.033 ± 0.048 a	1.113 ± 0.043 a	1.203 ± 0.003 ab
Media + PBS	0.751 ± 0.070 a	1.027 ± 0.115 a	1.079 ± 0.009 a	1.224 ± 0.016 a
Media + LPS	0.795 ± 0.043 a	1.098 ± 0.086 a	1.085 ± 0.057 a	1.144 ± 0.027 bc
LPS + SGE	0.816 ± 0.020 a	1.062 ± 0.030 a	1.154 ± 0.017 a	1.124 ± 0.006 c
LPS + PBS	0.712 ± 0.072 a	0.970 ± 0.016 a	1.142 ± 0.095 a	1.103 ± 0.032 c

Within individual columns, means followed by the same letter are not significantly different ($P \geq 0.05$).

Endothelial cells were seeded at 1 x 10^4 cells/well in a 96-well flat-bottomed tissue culture plate and cultured for 2 days, at which time a monolayer was formed. Monolayers were cultured with media, SGE, or PBS, with or without LPS for 4 to 24 hours. Cell viability was measured using MTS assay. Mean absorbance (OD ± S.E.M.) was measured using a microplate spectrophotometer.

Table 2. Constitutive expression of E-selectin by bEnd3 endothelial cells incubated with media, SGE, saliva, or PBS for 4 to 24 hours.

	Incubation period (hours)			
Treatment	**4**	**8**	**16**	**24**
Media	0.175 ± 0.005	0.173 ± 0.010	0.149 ± 0.008	0.173 ± 0.010
0.1 μg SGE	0.146 ± 0.009	0.189 ± 0.013	0.163 ± 0.013	0.153 ± 0.016
0.5 μg SGE	0.143 ± 0.011	0.165 ± 0.013	0.145 ± 0.009	0.137 ± 0.015 *
1.0 μg SGE	0.137 ± 0.008 *	0.166 ± 0.013	0.167 ± 0.016	0.135 ± 0.018 *
0.1 μg saliva	0.167 ± 0.017	0.187 ± 0.014	0.123 ± 0.006	0.131 ± 0.014 *
0.5 μg saliva	0.155 ± 0.014	0.184 ± 0.014	0.153 ± 0.008	0.135 ± 0.012 *
1.0 μg saliva	0.150 ± 0.011	0.167 ± 0.015	0.163 ± 0.017	0.142 ± 0.022 *
PBS	0.164 ± 0.011	0.158 ± 0.015	0.170 ± 0.018	0.143 ± 0.012

Asterisks indicate a significant difference ($P \leq 0.05$) in mean OD between treated and untreated (media alone) cells.

Endothelial cells were seeded at 1 x 10^4 cells/well in a 96-well flat-bottomed tissue culture plate and cultured for 2 days, at which time a monolayer was formed. Monolayers were cultured in the presence/absence of SGE, saliva, or PBS for 4 to 24 hours, and detection of cell surface expression of E-selectin was measured by whole-cell ELISA. Mean absorbance (OD ± S.E.M.) was measured using a microplate spectrophotometer.

dependent manner (0.1 μg = -12.6%; 0.5 μg = -21.7; 1.0 μg = -23.0%). At the higher concentrations of SGE (0.5 μg and 1.0 μg), the decreases in expression were significant; and, although not in a dose-dependent fashion, all concentrations of saliva significantly decreased the constitutive level of E-selectin expression after 24 hours. At all time points assayed, the constitutive level of E-selectin expressed by cells cultured with PBS did not significantly differ from the levels of expression reported for cells cultured with media alone.

Cells cultured with mrTNF-α had an average increase of 53% in the level of expression of E-selectin (0.257 ± 0.004), compared to constitutive levels from 4 to 24 hours. The mrTNF-α-stimulated levels of expression of E-selectin (mean OD ± S.E.M.) by cells cultured with media, SGE, saliva, or PBS are presented in Table 3. After 4 hours of culture with mrTNF-α, the stimulated level of expression of E-selectin (0.250 ± 0.021) was decreased in a dose-dependent manner when cells were incubated with SGE (0.1 μg = -7.6%; 0.5 μg = -14.4; 1.0 μg = -18.0%), but not saliva. The SGE-mediated suppression of the stimulated expression was significant at the higher concentrations (0.5 μg and 1.0 μg). The stimulated level of expression of E-selectin at 8 hours was significantly decreased when cells were incubated with 1.0 μg SGE (-12.4%), and only slightly decreased at the 0.1 μg (-4.3%) and 0.5 μg (-3.5%) concentrations. A dose-dependent effect was observed when cells were incubated with saliva, prior to the culture with mrTNF-α for 8 hours, resulting in differences of +3.9% (0.1 μg), -6.6% (0.5 μg), and -10.8% (1.0 μg), however, the level of expression was not significantly reduced at

Table 3. Murine recombinant TNF-α-induced expression of E-selectin by bEnd3 endothelial cells incubated with media, SGE, saliva, or PBS for 4 to 24 hours.

	Incubation period (hours)			
Treatment	**4**	**8**	**16**	**24**
Media	0.250 ± 0.021	0.259 ± 0.017	0.251 ± 0.016	0.268 ± 0.019
0.1 μg SGE	0.231 ± 0.015	0.248 ± 0.019	0.244 ± 0.014	0.228 ± 0.012 *
0.5 μg SGE	0.214 ± 0.012 *	0.250 ± 0.013	0.227 ± 0.014	0.230 ± 0.009 *
1.0 μg SGE	0.205 ± 0.011 *	0.227 ± 0.013 *	0.216 ± 0.007 *	0.201 ± 0.021 *
0.1 μg saliva	0.244 ± 0.018	0.269 ± 0.019	0.233 ± 0.011	0.238 ± 0.021
0.5 μg saliva	0.253 ± 0.011	0.242 ± 0.013	0.207 ± 0.012 *	0.231 ± 0.018 *
1.0 μg saliva	0.232 ± 0.012	0.231 ± 0.018	0.229 ± 0.013	0.232 ± 0.018 *
PBS	0.275 ± 0.009	0.250 ± 0.024	0.266 ± 0.024	0.244 ± 0.023

Asterisks indicate a significant difference ($P \leq 0.05$) in mean OD between treated and untreated (media alone) cells.

Endothelial cells were seeded at 1 x 10^4 cells/well in a 96-well flat-bottomed tissue culture plate and cultured for 2 days, at which time a monolayer was formed. Monolayers were cultured in the presence/absence of SGE, saliva, or PBS for 2 hours, then mrTNF-α for 4 to 24 hours, and detection of cell surface expression of E-selectin was measured by whole-cell ELISA. Mean absorbance (OD ± S.E.M.) was measured using a microplate spectrophotometer.

any concentration, compared to the cells incubated with mrTNF-α alone. Both SGE and saliva decreased the stimulated level of expression of E-selectin at 16 hours. The decreases in expression by cells cultured with SGE (0.1 μg = -2.8%; 0.5 μg = -9.6; 1.0 μg = -13.9%), but not saliva, occurred in a dose-dependent manner. Differences in expression of E-selectin by cell pre-treated with 1.0 μg SGE (0.216 ± 0.007) or 0.5 μg saliva (0.207 ± 0.012) were significant when compared to control cells. Although a distinct peak in the stimulated level of expression of E-selectin was not evident, the greatest level of suppression observed for cells pre-treated with SGE was observed at 24 hours. Similar to the levels of suppression observed for unstimulated cells at 24 hours, each concentration of SGE significantly decreased the stimulated levels of expression (0.1 μg = -15.0%; 0.5 μg = -14.2; 1.0 μg = -25.0%). Likewise, pre-treatment with saliva also significantly decreased the mrTNF-α-induced expression at the concentrations of both 0.5 μg (-13.8%) and 1.0 μg (-13.4%). Although the PBS treated cells resulted in a -9.0% decrease in the stimulated expression of E-selectin at the 24 hours, this suppression was less than those observed for cells treated with the SGE and saliva treatments, and non-significant. Furthermore, expression of E-selectin was not significantly affected when cells were pre-treated with PBS, before the addition of mrTNF-α, at any other time point assessed.

VCAM-1: The constitutive levels of expression of VCAM-1 (mean OD ± S.E.M.) by bEnd3 cells cultured with media, SGE, saliva, or PBS are provided in Table 4. Compared to the constitutive level of expression of E-selectin, VCAM-1 expression was either equal

Table 4. Constitutive expression of VCAM-1 by bEnd3 endothelial cells incubated with media, SGE, saliva, or PBS for 4 to 24 hours.

	Incubation period (hours)			
Treatment	**4**	**8**	**16**	**24**
Media	0.171 ± 0.024	0.196 ± 0.024	0.323 ± 0.039	0.362 ± 0.019
0.1 μg SGE	0.179 ± 0.023	0.194 ± 0.024	0.294 ± 0.038	0.319 ± 0.038
0.5 μg SGE	0.168 ± 0.024	0.144 ± 0.023	0.237 ± 0.028 *	0.278 ± 0.028 *
1.0 μg SGE	0.142 ± 0.025	0.126 ± 0.021 *	0.239 ± 0.034 *	0.277 ± 0.027 *
0.1 μg saliva	0.196 ± 0.022	0.209 ± 0.027	0.299 ± 0.033	0.361 ± 0.034
0.5 μg saliva	0.165 ± 0.026	0.156 ± 0.020	0.247 ± 0.028 *	0.304 ± 0.028 *
1.0 μg saliva	0.146 ± 0.024	0.144 ± 0.018	0.235 ± 0.034 *	0.284 ± 0.029 *
PBS	0.223 ± 0.025	0.168 ± 0.021	0.288 ± 0.040	0.351 ± 0.019

Asterisks indicate a significant difference ($P \leq 0.05$) in mean OD between treated and untreated (media alone) cells.

Endothelial cells were seeded at 1 x 10^4 cells/well in a 96-well flat-bottomed tissue culture plate and cultured for 2 days, at which time a monolayer was formed. Monolayers were cultured in the presence/absence of SGE, saliva, or PBS for 4 to 24 hours, and detection of cell surface expression of VCAM-1 was measured by whole-cell ELISA. Mean absorbance (OD ± S.E.M.) was measured using a microplate spectrophotometer.

to, or higher, throughout the 24 hour period. The constitutive levels of expression of VCAM-1 at 4 (0.171 ± 0.024), 8 (0.196 ± 0.024), 16 (0.323 ± 0.039), and 24 (0.362 ± 0.019) hours were decreased in a dose-dependent manner when cells were incubated with either SGE or saliva. Significant decreases were observed for the 0.5 μg concentration of SGE at 16 (-26.6%) and 24 (-23.4%) hours. Also, significant decreases were observed with the 1.0 μg concentration of SGE at 8 (-35.7%), 16 (-26.0%), and 24 (-23.5%) hours. Similar to the decreased levels of expression observed for cells cultured with SGE, saliva significantly suppressed the constitutive level of VCAM-1 expression at 16 (0.5 μg = -23.5%; 1.0 μg = -27.3%) and 24 (0.5 μg = -16.0%; 1.0 μg = -21.6%) hours. The cells used as controls for the tick protein, the PBS alone treatment, varied slightly compared to the media alone treatments; however, the differences in expression between control cells cultured with PBS versus those cultured with media alone were not significant.

The constitutive level of expression of VCAM-1 was upregulated throughout the 24 hour period when endothelial cells were cultured with *E.coli* LPS. In Table 5, the *E.coli* LPS-stimulated levels of expression of VCAM-1 (mean OD ± S.E.M.) by cells cultured with media, SGE, saliva, or PBS are provided. A dose-dependent effect was observed when cells were incubated with varying concentrations of either SGE or saliva at 4 and 8 hours, however, only the higher concentrations of both SGE and saliva reduced the level of expression, compared to cells incubated with LPS alone. Of the decreases observed at both 4 and 8 hours, only the 1.0 μg concentration of saliva significantly suppressed the LPS-induced expression at 8 hours (-16.2%). At 16 hours, all

Table 5. *E.coli* LPS-induced expression of VCAM-1 by bEnd3 endothelial cells incubated with media, SGE, saliva, or PBS for 4 to 24 hours.

	Incubation period (hours)			
Treatment	**4**	**8**	**16**	**24**
Media	0.303 ± 0.033	0.415 ± 0.033	0.524 ± 0.047	0.530 ± 0.032
0.1 μg SGE	0.372 ± 0.041	0.440 ± 0.034	0.491 ± 0.050	0.489 ± 0.034
0.5 μg SGE	0.311 ± 0.044	0.407 ± 0.044	0.417 ± 0.040 *	0.428 ± 0.032 *
1.0 μg SGE	0.294 ± 0.041	0.358 ± 0.031	0.448 ± 0.057 *	0.436 ± 0.043 *
0.1 μg saliva	0.342 ± 0.041	0.445 ± 0.049	0.510 ± 0.067	0.538 ± 0.037
0.5 μg saliva	0.288 ± 0.038	0.382 ± 0.039	0.456 ± 0.050	0.466 ± 0.049 *
1.0 μg saliva	0.287 ± 0.041	0.348 ± 0.045 *	0.486 ± 0.062	0.450 ± 0.052 *
PBS	0.469 ± 0.053 *	0.439 ± 0.028	0.595 ± 0.027	0.528 ± 0.031

Asterisks indicate a significant difference ($P \leq 0.05$) in mean OD between treated and untreated (media alone) cells.

Endothelial cells were seeded at 1 x 10^4 cells/well in a 96-well flat-bottomed tissue culture plate and cultured for 2 days, at which time a monolayer was formed. Monolayers were cultured in the presence/absence of SGE, saliva, or PBS for 2 hours, then LPS for 4 to 24 hours, and detection of cell surface expression of VCAM-1 was measured by whole-cell ELISA. Mean absorbance (OD ± S.E.M.) was measured using a microplate spectrophotometer.

concentrations of both SGE and saliva reduced the stimulated level of expression VCAM-1, with significant reductions observed with the 0.5 μg (-20.4%) and 1.0 μg (-14.5%) concentrations of SGE. The LPS-stimulated level of expression at 24 hours (0.530 ± 0.032) was decreased when cells were pre-treated with SGE (0.1 μg = -7.7%; 0.5 μg = -19.3%; 1.0 μg = -17.7%) or the higher concentrations of saliva (0.5 μg = -12.1%; 1.0 μg = -15.1%). Suppression observed at the higher concentrations of SGE and saliva, 0.5 μg and 1.0 μg, was significant. VCAM-1 levels for cells cultured with PBS were significantly higher (+54.8%) at 4 hours, compared to cells incubated with LPS and media alone, however cells cultured with PBS for 8 through 24 hours did not significantly differ from absorbancies reported for cells cultured with LPS and media alone.

ICAM-1: The constitutive levels of expression of ICAM-1 (mean OD ± S.E.M.) by bEnd3 cells cultured with media, SGE, saliva, or PBS are presented in Table 6. Compared to both E-selectin and VCAM-1 expression by these endothelial cells, the constitutive level of ICAM-1 expression was very much lower during the 24 hour incubation period. The constitutive level of expression of ICAM-1 at 4 hours (0.020 ± 0.005) was decreased when cells were incubated with SGE or saliva. At the 0.5 μg concentrations of both SGE and saliva the decreases in expression of ICAM-1 were significant (SGE = -80.0%; saliva = -95.0%), essentially blocking expression of ICAM-1. At 8 hours, the constitutive level of expression (0.019 ± 0.006) was decreased when cells were cultured with varying concentrations of both SGE and saliva. The decreases in expression observed when cells were cultured with SGE, but not saliva were significant.

Table 6. Constitutive expression of ICAM-1 by bEnd3 endothelial cells incubated with media, SGE, saliva, or PBS for 4 to 24 hours.

	Incubation period (hours)			
Treatment	**4**	**8**	**16**	**24**
Media	0.020 ± 0.005	0.019 ± 0.006	0.020 ± 0.007	0.053 ± 0.014
0.1 μg SGE	0.008 ± 0.005	0.005 ± 0.007 *	0.015 ± 0.006	0.037 ± 0.015
0.5 μg SGE	0.004 ± 0.004 *	0.004 ± 0.002 *	0.008 ± 0.006	0.038 ± 0.013
1.0 μg SGE	0.008 ± 0.003	0.005 ± 0.004 *	0.008 ± 0.006	0.039 ± 0.010
0.1 μg saliva	0.013 ± 0.007	0.010 ± 0.006	0.010 ± 0.004	0.050 ± 0.012
0.5 μg saliva	0.001 ± 0.005 *	0.007 ± 0.002	0.004 ± 0.005 *	0.055 ± 0.011
1.0 μg saliva	0.008 ± 0.006	0.011 ± 0.003	0.009 ± 0.006	0.052 ± 0.013
PBS	0.021 ± 0.006	0.014 ± 0.003	0.027 ± 0.008	0.097 ± 0.022 *

Asterisks indicate a significant difference ($P \leq 0.05$) in mean OD between treated and untreated (media alone) cells.

Endothelial cells were seeded at 1 x 10^4 cells/well in a 96-well flat-bottomed tissue culture plate and cultured for 2 days, at which time a monolayer was formed. Monolayers were cultured in the presence/absence of SGE, saliva, or PBS for 4 to 24 hours, and detection of cell surface expression of ICAM-1 was measured by whole-cell ELISA. Mean absorbance (OD ± S.E.M.) was measured using a microplate spectrophotometer.

Constitutive expression of ICAM-1 at 16 hours (0.020 ± 0.007) was decreased when cells were cultured with either SGE or saliva at all concentrations used, however, only the 0.5 μg concentration of saliva significantly decreased expression. After 24 hours of culture, the constitutive level of expression of ICAM-1 (0.053 ± 0.014) was greater than that observed from 4 to 16 hours. Although neither SGE nor saliva significantly modulated the expression of ICAM-1 after 24 hours of culture, cells cultured with PBS alone had significantly increased expression, compared to cells cultured in media alone. The level of expression of ICAM-1 by cells cultured with PBS for 4 through 16 hours did not significantly differ from the levels observed for cells cultured with media alone.

The mrTNF-α-stimulated levels of expression of ICAM-1 (mean OD ± S.E.M.) by bEnd3 cells cultured with media, SGE, saliva, or PBS are shown in Table 7. A significant upregulation of ICAM-1, compared to constitutive levels, was observed after 16 (+105%) and 24 (+134%) hours when cells were cultured with mrTNF-α. The stimulated level of expression of ICAM-1 at 4 hours (0.027 ± 0.004) was significantly decreased when cells were incubated with either 0.1 μg (-77.8%) or 1.0 μg (-81.5%) of SGE, and all concentrations of saliva (0.1 μg = -70.4%; 0.5 μg = -96.3%; 1.0 μg = -81.5%). At 8 hours, the stimulated level of expression (0.030 ± 0.009) was significantly decreased when cells were incubated with 0.5 μg or 1.0 μg of either SGE (0.5 μg = -53.3%; 1.0 μg = -73.3%) or saliva (0.5 μg = -46.7%; 1.0 μg = -40.0%). Both SGE and saliva decreased the mrTNF-α-stimulated level of expression of ICAM-1 at 16 hours. Significant decreases were observed when cells were cultured with SGE (0.1 μg = -39.0%; 0.5 μg = -31.7%; 1.0 μg = -53.7%). Cells pre-treated with saliva showed a

Table 7. Murine recombinant TNF-α-induced expression of ICAM-1 by bEnd3 endothelial cells incubated with media, SGE, saliva, or PBS for 4 to 24 hours.

	Incubation period (hours)			
Treatment	**4**	**8**	**16**	**24**
Media	0.027 ± 0.004	0.030 ± 0.009	0.041 ± 0.008	0.124 ± 0.025
0.1 μg SGE	0.006 ± 0.004 *	0.020 ± 0.006	0.025 ± 0.009 *	0.082 ± 0.023 *
0.5 μg SGE	0.020 ± 0.011	0.014 ± 0.006 *	0.028 ± 0.009 *	0.072 ± 0.018 *
1.0 μg SGE	0.005 ± 0.005 *	0.008 ± 0.006 *	0.019 ± 0.011 *	0.068 ± 0.019 *
0.1 μg saliva	0.008 ± 0.005 *	0.026 ± 0.009	0.032 ± 0.009	0.087 ± 0.021 *
0.5 μg saliva	0.001 ± 0.005 *	0.016 ± 0.005 *	0.024 ± 0.012 *	0.076 ± 0.023 *
1.0 μg saliva	0.005 ± 0.005 *	0.018 ± 0.004 *	0.015 ± 0.008 *	0.074 ± 0.023 *
PBS	0.021 ± 0.006	0.031 ± 0.005	0.052 ± 0.013	0.157 ± 0.033

Asterisks indicate a significant difference ($P \leq 0.05$) in mean OD between treated and untreated (media alone) cells.

Endothelial cells were seeded at 1 x 10^4 cells/well in a 96-well flat-bottomed tissue culture plate and cultured for 2 days, at which time a monolayer was formed. Monolayers were cultured in the presence/absence of SGE, saliva, or PBS for 2 hours, then mrTNF-α for 4 to 24 hours, and detection of cell surface expression of ICAM-1 was measured by whole-cell ELISA. Mean absorbance (OD ± S.E.M.) was measured using a microplate spectrophotometer.

dose-dependent decrease in expression (0.1 μg = -22.0%; 0.5 μg = -41.5%; 1.0 μg = -63.4%), with the suppression in the level of expression for the 0.5 μg and 1.0 μg concentrations being significant. Incubation with mrTNF-α resulted in a peak level of expression of ICAM-1 (0.124 ± 0.025) at 24 hours. The stimulated level of expression was significantly reduced in a dose-dependent manner when cells were cultured with either SGE (0.1 μg = -33.9%; 0.5 μg = -41.9%; 1.0 μg = -45.2%) or saliva (0.1 μg = -29.8%; 0.5 μg = -38.7%; 1.0 μg = -40.3%). Culturing cell with PBS alone, before the addition of mrTNF-α did not significantly affect the expression of ICAM-1, compared to cells cultured with media alone, prior to the addition of mrTNF-α.

Lymphocyte Expression of Adhesion Molecules *in vitro*

The constitutive and Con A-induced levels of expression of LFA-1 and VLA-4 (mean OD ± S.E.M.) by tick-naive BALB/c lymphocytes cultured with media, SGE, saliva, or PBS are presented in Table 8.

LFA-1: A non-significant trend towards suppression in the level of expression of LFA-1 cultured with media alone (1.170 ± 0.060) was observed when cells were cultured with varying concentrations of either SGE (0.1 μg = -12.1%; 0.5 μg = -8.6%; 1.0 μg = -2.3%) or saliva (0.1 μg = -9.3%; 0.5 μg = -12.7; 1.0 μg = -12.5) for 48 hours. Lymphocytes cultured in the presence of Con A for 48 hours had a +48.6% increase in expression of LFA-1, compared to lymphocytes cultured in media alone. Pre-incubation for 2 hours with SGE or saliva, before the addition of Con A, decreased levels of Con A-induced expression of LFA-1. Significant decreases in the level of expression were observed when cell were pre-treated with each concentration of SGE (0.1 μg = -25.2%; 0.5 μg =

Table 8. Constitutive and Con A-induced expression of LFA-1 and VLA-4 by lymphocytes from BALB/c mice incubated with media, SGE, saliva, or PBS for 48 hours.

	LFA-1		VLA-4	
Treatment	**Constitutive**	**Con A-induced**	**Constitutive**	**Con A-induced**
Media	1.170 ± 0.060	1.739 ± 0.083	0.686 ± 0.024	0.646 ± 0.049
0.1 μg SGE	1.029 ± 0.076	1.300 ± 0.179 *	0.696 ± 0.037	0.498 ± 0.080
0.5 μg SGE	1.070 ± 0.070	1.032 ± 0.274 *	0.769 ± 0.063	0.463 ± 0.084
1.0 μg SGE	1.143 ± 0.095	1.315 ± 0.135 *	0.760 ± 0.057	0.493 ± 0.058
0.1 μg saliva	1.061 ± 0.095	1.540 ± 0.147	0.635 ± 0.078	0.603 ± 0.072
0.5 μg saliva	1.022 ± 0.065	1.568 ± 0.293	0.665 ± 0.035	0.782 ± 0.163
1.0 μg saliva	1.024 ± 0.055	1.462 ± 0.195 *	0.635 ± 0.022	0.676 ± 0.113
PBS	1.285 ± 0.126	1.835 ± 0.147	0.883 ± 0.097 *	0.836 ± 0.095 *

Asterisks indicate a significant difference ($P \leq 0.05$) in mean OD between treated and untreated (media alone) cells.

Lymphocytes were seeded at 5 x 10^6 cells/well in a 24-well flat-bottomed tissue culture plate and cultured with media, SGE, saliva, or PBS for 2 hours. Con A was added to specific wells, and cells were cultured another 46 to 48 hours. Detection of cell surface expression of LFA-1 and VLA-4 was measured by whole-cell ELISA. Mean absorbance (OD ± S.E.M.) was measured using a microplate spectrophotometer.

-40.7%; 1.0 μg = -24.4%), or 1.0 μg saliva (-15.9%). Being cultured with PBS, or pretreatment with PBS prior to the addition of Con A, did not decrease the levels of expression of LFA-1.

VLA-4: The level of expression of VLA-4 cultured with media alone (0.686 ± 0.024) was slightly enhanced when cells were cultured with varying concentrations of SGE, but not to a significant level. Incubation of cells with saliva showed a trend towards suppressed levels of expression (0.1 μg = -7.4%; 0.5 μg = -3.1%; 1.0 μg = -7.4%), however, these differences were not significant. Incubation of lymphocytes with Con A did not increase in expression of VLA-4 (0.646 ± 0.049). Pre-incubation for 2 hours with SGE, before the addition of Con A, resulted in decreased levels of expression of VLA-4, compared to those observed for cells incubated with Con A alone. However, pre-treatment with saliva did not decrease the level of expression observed for cells incubated with Con A alone. For lymphocytes cultured in both the presence or absence of Con A, PBS significantly increases the levels of expression of VLA-4.

Tick Infestation

A total of 115 ticks were recovered from 13 Group 1 mice, infested once, resulting in an average of 8.85 ticks per mouse. For Group 2 mice (13 infested), 110 ticks were recovered after the first infestation, with an average of 8.46 ticks per mouse. After the second infestation, 103 ticks were recovered, resulting in an average of 7.92 ticks per mouse. Significant differences in parasite load between infestations were not observed.

Tick Biology

Mean weight (mg) and viability (percent that molt to adult) of ticks recovered after one or two infestations are presented in Table 9. No differences were observed in mean weight or viability of nymphal ticks after the first infestation of either Group 1 or Group 2 mice. However, ticks recovered from Group 2 mice after the second infestation had significantly reduced mean engorgement weight (-35.7%), and decreased viability (-19.5%), compared to ticks recovered from once infested mice of both Group 1 and Group 2.

Lymphocyte Expression of Adhesion Molecules by Cells Derived from Infested Mice

The constitutive and Con A-induced levels of expression of LFA-1 and VLA-4 (mean OD ± S.E.M.) by lymphocytes from uninfested controls and infested BALB/c mice are presented in Table 10. After first or second infestations, lymphocytes recovered from infested mice had decreased levels of both constitutive and Con A-induced expression of LFA-1, when compared to cells of uninfested controls. The constitutive and Con A-induced levels of expression of LFA-1 were significantly decreased (constitutive = -13.5%; Con A-induced = -27.2%) for infested mice after the first exposure. After the second exposure, the level of suppression of LFA-1 for infested animals was reduced (constitutive = -3.1%; Con A-induced = -11.6%). However, this infestation-related decrease in Con A-stimulated LFA-1 expression, after the second infestation, was significant.

Table 9. Biology parameters, mean weight (mg) and percent molt, of ticks recovered from BALB\c mice infested once (Group 1) or twice (Group 2).

Infestation/Parameters	First infestation	Second infestation
Group 1		
Mean tick weight ± S.E.M.	16.9 ± 1.4	-
Percent molt ± S.E.M.	82.9 ± 3.5	-
Group 2		
Mean tick weight ± S.E.M.	16.8 ± 1.0	10.8 ± 1.7*
Percent molt ± S.E.M.	82.5 ± 4.1	66.4 ± 9.8

Asterisk indicates a significant difference ($P \leq 0.05$) in mean tick engorgement weight between first and second infestations.

Balb/c mice were infested with *D. andersoni* nymphs one or two times. Nymphs were allowed to feed to engorgement, or a maximum of seven days, which ever came first. When ticks detached, or on the seventh day, they were weighed and then maintained in a humidified environment. Ability to molt was monitored visually following feeding, and percent molt was calculated by dividing the number of ticks recovered from each group of mice by the number of ticks that successfully molted to adult life stage.

Table 10. Constitutive and Con A-induced expression of LFA-1 and VLA-4 by lymphocytes from BALB/c mice infested one or two times with 10 *D. andersoni* nymphs.

Adhesion molecule	First Infestation		Second Infestation	
	Control	Infested	Control	Infested
LFA-1				
Constitutive	0.929 ± 0.052	0.804 ± 0.060*	1.465 ± 0.067	1.420 ± 0.048
Con A-induced	1.598 ± 0.044	1.164 ± 0.064*	1.937 ± 0.057	1.712 ± 0.065*
VLA-4				
Constitutive	0.637 ± 0.029	0.636 ± 0.047	1.156 ± 0.072	1.020 ± 0.036*
Con A-induced	0.771 ± 0.037	0.639 ± 0.039*	0.911 ± 0.063	0.809 ± 0.055*

Asterisks indicate a significant difference ($P \leq 0.05$) in mean OD between infested and control (uninfested) mice.

Lymphocytes from infested and uninfested control mice were seeded at 5 x 10^6 cells/well in a 24-well flat-bottomed tissue culture plate and cultured with Con A for 48 hours. Detection of cell surface expression of LFA-1 and VLA-4 was measured by whole-cell ELISA. Mean absorbance (OD ± S.E.M.) was measured using a microplate spectrophotometer.

The constitutive level of expression of VLA-4 by lymphocytes was unchanged in infested mice, compared to uninfested controls, after the first exposure. After the second infestation, the constitutive level of expression of VLA-4 by lymphocytes from tick-exposed mice was significantly reduced (-11.8%) compared to cells from uninfested controls. Compared to constitutive levels in control mice, Con A stimulated an increased level of expression of VLA-4 after the first infestation, but not after the second exposure. Con A-induced expression of VLA-4 by lymphocytes was significantly decreased for infested mice after both the first (-17.1%) and, to a lesser extent, the second (-11.2%) infestations.

Mitogen-Induced *in vitro* Splenocyte Proliferation

Splenocytes recovered from uninfested controls and infested mice were tested for responsiveness to the T cell mitogen Con A. For each mouse, a stimulation index was calculated by dividing mean counts per minute (CPM) obtained for different treatments of Con A by the mean CPM obtained for cells incubated with media alone. For each group of mice, control and once or twice infested, stimulation indices were combined and a mean was calculated. Data for control and infested mice are presented in Table 11 as mean stimulation indices (± S.E.M.). The actual CPM ± S.E.M. for each group of mice are presented in a similar manner in Appendix II (Table A).

Stimulation indices of splenocytes of mice infested either once or twice prior to exposure to Con A were decreased when compared to uninfested controls treated in a similar manner. After the first infestation, stimulation indices of infested mice were 26% (0.5 μg Con A), 21% (1.0 μg Con A), and 33% (2.0 μg Con A) lower than that of

Table 11. ***In vitro*** **proliferative responses of splenocytes from BALB/c mice, infested one or two times with 10** ***D. andersoni*** **nymphs, to Concanavalin A (Con A).**

Infestation/ Group	Treatment		
	0.5 μg Con A	1.0 μg Con A	2.0 μg Con A
First Infestation			
Control	202.1 ± 20.8	198.2 ± 19.8	172.5 ± 16.3
Infested	149.6 ± 8.0 *	156.2 ± 25.7	115.2 ± 5.5 *
Second Infestation			
Control	235.4 ± 23.1	231.0 ± 22.6	211.7 ± 21.6
Infested	202.3 ± 19.7	190.8 ± 21.0 *	160.7 ± 18.1 *

Asterisks indicate a significant difference ($P \leq 0.05$) in mean SI between infested and control (uninfested) mice.

5×10^5 splenocytes from infested and control mice were incubated with varying concentrations of Con A for 54 hours and then pulsed with one μCi of ^{3}H-thymidine for 17 hours. Cells were harvested and radioactivity incorporation was determined by counting in liquid scintillation counter. Data are presented as mean stimulation indices (± S.E.M.). Stimulation indices were calculated by dividing the mean CPM obtained from different treatment wells by mean CPM obtained from control wells (cells incubated with media not containing Con A).

controls. At the 2.0 and 0.5 μg/well concentration of Con A, the responses were significantly lower than those of control mice. Upon a second infestation, stimulation indices were 14% (0.5 μg Con A), 17% (1.0 μg Con A), and 24% (2.0 μg Con A) lower than those of control mice. At the higher concentrations of Con A (2.0 μg/well and 1.0 μg/well), the decreases in stimulation indices were significant. For each concentration of Con A, mice that had been exposed to ticks twice were consistently less suppressed than cells from mice infested only once. At the lowest concentration of Con A (0.5 μg/well) tested, the stimulation index for the second infestation (202.3 ± 19.7) was significantly higher than that, for the same concentration, during the first infestation (149.6 ± 8.0).

Antigen-Induced *in vitro* Splenocyte Proliferation

Splenocytes recovered from uninfested controls and infested mice were tested for responsiveness to tick-derived salivary gland antigens. For each mouse, a stimulation index is calculated by dividing mean counts per minute (CPM) obtained for different treatments of either SGE or saliva by the mean CPM obtained for cells incubated with PBS not containing tick antigen. For each group of mice, control and once or twice infested, stimulation indices were combined and a mean was calculated. Data for control and infested mice are presented in Table 12 (SGE) and Table 13 (saliva) as mean stimulation indices (± S.E.M.). The actual CPM ± S.E.M. for each group of mice are presented in Appendix II (Table B, SGE; Table C, saliva).

Splenocytes from uninfested control and infested mice were incubated with either *D. andersoni* SGE or saliva, to examine tick salivary gland antigen-driven responses. A dose-dependent increase in the stimulation indices was observed for cells, recovered from

Table 12. *In vitro* proliferative responses of splenocytes from BALB/c mice, infested one or two times with 10 *D. andersoni* nymphs, to salivary gland extract (SGE).

Infestation/ Group	Treatment		
	0.1 μg SGE	0.5 μg SGE	1.0 μg SGE
First Infestation			
Control	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
Infested	2.5 ± 0.5	4.6 ± 0.6 *	6.3 ± 1.0 *
Second Infestation			
Control	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
Infested	2.2 ± 0.3	5.8 ± 0.9 *	7.1 ± 0.9 *

Asterisks indicate a significant difference ($P \leq 0.05$) in mean SI between infested and control (uninfested) mice.

5 x 10^5 splenocytes from infested and control mice were incubated with varying concentrations of SGE for 54 hours and then pulsed with one μCi of ^{3}H-thymidine for 17 hours. Cells were harvested and radioactivity incorporation was determined by counting in liquid scintillation counter. Data are presented as mean stimulation indices (± S.E.M.). Stimulation indices were calculated by dividing the mean CPM obtained from different treatment wells by mean CPM obtained from control wells (cells incubated with PBS not containing SGE).

Table 13. *In vitro* proliferative responses of splenocytes from BALB/c mice, infested one or two times with 10 *D. andersoni* nymphs, to saliva.

Infestation/ Group	Treatment		
	0.1 μg saliva	0.5 μg saliva	1.0 μg saliva
First Infestation			
Control	0.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
Infested	1.1 ± 0.1	2.3 ± 0.2 *	2.8 ± 0.0 *
Second Infestation			
Control	0.9 ± 0.1	0.9 ± 0.0	0.9 ± 0.0
Infested	1.2 ± 0.0	2.4 ± 0.4 *	2.9 ± 0.1 *

Asterisks indicate a significant difference ($P \leq 0.05$) in mean SI between infested and control (uninfested) mice.

5×10^5 splenocytes from infested and control mice were incubated with varying concentrations of saliva for 54 hours and then pulsed with one μCi of ^{3}H-thymidine for 17 hours. Cells were harvested and radioactivity incorporation was determined by counting in liquid scintillation counter. Data are presented as mean stimulation indices (± S.E.M.). Stimulation indices were calculated by dividing the mean CPM obtained from different treatment wells by mean CPM obtained from control wells (cells incubated with PBS not containing saliva).

stimulation indices were observed when splenocytes from infested mice were cultured with the higher concentrations of SGE (0.5 μg/well = 5.4-fold increase; 1.0 μg/well = 6.9-fold increase), but not the lowest concentration (0.1 μg/well = 1.4-fold increase). The differences in stimulation indices between infestations were not significant.

Increased *in vitro* proliferative responsiveness of splenocytes from infested mice to tick saliva was observed over a range of saliva concentrations. Incubation of splenocytes in the presence of saliva resulted in a 2.5-fold increase, at the 1.0 μg/well concentration, in the stimulation indices of infested mice, compared to uninfested controls. The 0.5 μg/well concentration resulted in a 1.6-fold increase, while a 0.6-fold increase in the stimulation index was observed for lymphocytes from infested mice cultured with 0.1 μg of saliva per well. At the two higher concentrations, the increase in stimulation indices were significant. Saliva had a similar effect on lymphocytes from mice that were infested twice. Increases in stimulation indices were 0.3-fold (0.1 μg/well), 1.6-fold (0.5 μg/well), and 2.2-fold (1.0 μg/well), with the two higher concentrations of saliva resulting in a significant increase.

T lymphocyte Production of Cytokines

The production of T lymphocyte cytokines by uninfested controls and infested BALB/c mice (mean ng/ml ± S.E.M.), are shown in Table 14. For each mouse, concentrations of cytokines were determined. Within each group of mice, control and infested one or two times, cytokine concentrations were combined, and a mean was calculated

Table 14. Concentration in ng/ml of cytokines produced by Con A-stimulated lymphocytes from BALB/c mice infested one or two times with 10 *D. andersoni* nymphs.

	First Infestation†		Second Infestation†	
Cytokine	**Control**	**Infested**	**Control**	**Infested**
Th 1				
IL-2*	22.4 ± 0.9 b	23.0 ± 1.4 b	26.2 ± 1.3 a	19.0 ± 1.1 c
IFN-γ	346.6 ± 36.0 b	403.1 ± 48.2 a	303.4 ± 16.1 b	313.8 ± 26.1 b
Th 2				
IL-4*	4.5 ± 0.5 c	13.3 ± 0.7 b	2.9 ± 0.3 d	16.0 ± 0.8 a
IL-10*	15.3 ± 0.8 c	33.4 ± 3.1 b	11.5 ± 0.9 c	52.0 ± 3.4 a

"†" indicates that for each cytokine, means followed by the same letter are not statistically different ($P \geq 0.05$). Asterisks indicate a significant difference ($P \leq 0.05$) in the ratio (infested mice versus uninfested control mice) of cytokine production between infestations.

Lymphocytes from infested and uninfested control mice were seeded at 2.5 x 10^6 cells/well in a 24-well flat-bottomed tissue culture plate and cultured with Con A for 48 hours. Concentration of cytokines in supernatant determined by antigen-capture ELISA.

After either one or two infestations, significant differences were observed between lymphocytes from uninfested and infested mice in their abilities to produce Th1 and Th2 cytokines. After the first infestation, production of Th 1 cytokines IL-2 and IFN-γ was greater for cells from infested mice than for similar cells from uninfested controls, with production of IFN-γ by infested mice significantly increased (+16.3%). Following the second infestation, IL-2 production was significantly decreased (-27.6%), and IFN-γ production was slightly increased (+3.4%). A significant decrease in the ability of lymphocytes to produce IL-2 (-17.4%) was observed for twice infested mice, when compared to mice infested only one time. However, compared to controls, the ability of lymphocytes from twice infested mice to produce IFN-γ was unchanged. T-lymphocyte production of Th2 cytokines, IL-4 and IL-10, was significantly increased after both the first infestation (IL-4 = +194.4%; IL-10 = +117.9%), and the second infestation (IL-4 = +449.8%; IL-10 = +353.2%). In addition, Th2 cytokine production was significantly increased when the second infestation was compared to the first exposure to *D. andersoni* nymphs.

CHAPTER IV

DISCUSSION

In the present study, the ability of *D. andersoni* SGE and saliva to modulate the expression of endothelial cell adhesion molecules was assessed. Through co-culture of endothelial monolayers, or pre-culture in the case of stimulated cells, with SGE or saliva, significant down regulation of endothelial cell expression of E-selectin, VCAM-1, and ICAM-1 was observed. In addition to endothelial cell adhesion molecule regulation, lymphocyte expression of the integrins LFA-1 and VLA-4 was also suppressed when cells were exposed to either tick SGE or saliva. *In vitro* assays to determine the regulation of immune mechanisms by tick salivary gland molecules are critically important initial steps in characterizing the relationship between ticks and their hosts. An important aspect of this study was the ability to correlate *in vitro* responses to similar responses observed in mice infested with ticks. Infestation of BALB/c mice with nymphal *D. andersoni* ticks resulted in the suppression of lymphocyte integrins in infested mice, compared to uninfested control mice. Furthermore, T lymphocyte responses, which likely are important for the expression of acquired resistance, were also described. A decrease in the magnitude of suppression of mitogen responsiveness, combined with an increase in lymphocyte recognition of tick-derived antigen, during successive infestations correlate to the expression of acquired resistance observed in this study. The cytokine production and subsequent changes in the profile reported in the present study is the first description of T lymphocyte cytokines in a host that develops acquired resistance to tick feeding.

Complex immunological interactions occur at the tick-host interface (Wikel, 1996d). Tick feeding stimulates a number of host immune mechanisms involving antibodies, complement, mast cells, basophils, eosinophils, antigen-presenting cells, lymphocytes, and cytokines (Wikel, 1996a; 1996d; 1999). Despite this vigorous host immune response to feeding, an ixodid tick is typically capable of extracting blood from the host over a period of days. Successful tick feeding is achieved through counteracting host defenses, including host cytokines, antibody production, complement, and T cell mediated immune responses (Wikel and Bergman, 1997). Modulation of host immunity is linked to tick salivary gland extracts (Ramachandra and Wikel, 1992) and infestation (Schoeler et al., 1999). Furthermore, a 36 kDa protein in the salivary glands of female *D. andersoni* was shown to suppress *in vitro* T lymphocyte responsiveness to Con A (Bergman et al., 1998).

Murine endothelial cells are useful for examining the regulation and temporal expression of adhesion molecules *in vitro.* The ability to measure cell viability and growth is important in identifying cytotoxic effects that may result in limited growth or even death of cells used in the assay. In this study, the growth of endothelial cells cultured with salivary gland-derived molecules was assessed throughout the incubation period used to determine the influence of tick protein on adhesion molecule expression. In addition to SGE, the impact of PBS on cellular growth was also determined to insure the media in which tick protein was diluted did not adversely affect cell viability. Interestingly, after 24 hours, culture with either SGE or PBS significantly enhanced cell viability, compared to cells cultured with media alone. This may be explained by the

presence of stimulatory molecules present in SGE that are capable of inducing endothelial cell proliferation. Although this does not clearly explain the similar observation for cells cultured with PBS, it is important to note that neither PBS or SGE diluted in PBS negatively impacted cellular growth. Based on these observations, it can concluded that the SGE-induced decrease of adhesion molecules was not due to cell death.

Induction of E-selectin, VCAM-1, and ICAM-1 on murine endothelial cells have similar kinetics to those reported for human primary endothelial cells (Hahne et al., 1993). In the present study, E-selectin was found to be constitutively expressed, and inducible with mrTNF-α, on the bEnd3 endothelial cells. Similar results were obtained for the same cell line, when constitutive and induced E-selectin expression were examined using whole-cell ELISA (Hahne et al., 1993) and flow cytometric analysis (Henseleit et al., 1996). Constitutive and LPS-induced expression of VCAM-1 by bEnd3 cells was consistent with previous studies (Hahne et al., 1993). Additionally, the constitutive expression of ICAM-1 was minimal throughout the 24 hour period measured, with a low level of ICAM-1 expressed, when cultured with mrTNF-α. These findings for ICAM-1 are consistent with the results of others, using the same cell line (Hahne et al., 1993; Henseleit et al., 1996).

Although this is the first report of tick modulation of endothelial adhesion molecules *in vitro*, others have examined the influence of tick feeding on adhesion molecules in the skin. Mbow et al. (1994b) reported an increase in ICAM-1 expression, observed by immunostaining, in BALB\c mice repeatedly infested with *I. ricinus* nymphs. While expression of ICAM-1 was reported as weak on vascular endothelial cells, 72

hours after tick attachment, it increased as ticks were infested a second and third time (Mbow et al., 1994b).

An inflammatory response involves stimulus-driven cellular migration to the specific site of injury or infection (Springer, 1995). This migration is due, in part, to the interactions of LFA-1, present on most leukocytes, with ICAM-1,2; and VLA-4, which is present on B and T lymphocytes, macrophages, basophils, eosinophils and NK cells, with VCAM-1 (Springer, 1995). The present study identifies a new mechanism by which ticks modulate host inflammatory responses. Through down regulation of adhesion molecule expression on both endothelium and lymphocytes, ticks could decrease cellular traffic to feeding sites. Decreased infiltrates of cells responsible for anti-tick immunity will increase the likelihood of successful bloodmeal acquisition by feeding ticks.

A number of infectious agents interact with and/or modulate cellular adhesion molecules as an important component of disease pathogenesis (Kerr, 1999). One example is the interaction of tick-transmitted *B. burgdorferi* with endothelial cells, specifically the ability of such organisms to induce adhesion molecules expression (Boggemeyer et al., 1994; Sellati et al., 1995). *Borrelia burgdorferi* activates endothelial cells to express E-selectin, VCAM-1, and ICAM-1, and promotes transendothelial migration of neutrophils (Sellati et al., 1995). Likewise, an increase in neutrophil adhesion due to leukocyte interaction with E-selectin was observed 6 to 8 hours after infection of endothelial cells with *R. rickettsii,* causative agent of Rocky Mountain spotted fever, contributing to the pathologic changes observed with this disease (Sporn et al., 1993). Studies examining pathogen-endothelial cell interaction are important in

determining the pathology of disease, however, there is an additional need to examine the vector influence on pathogen transmission and establishment.

In this study the observation that tick-derived salivary gland molecules can downregulate endothelial cell adhesion molecule expression could be important in pathogen establishment. Because the interaction of many tick-transmitted pathogens with endothelial cells results in the increased expression of adhesion molecules, tick suppression of adhesion molecule expression, in part provides a immunologically privileged site in which pathogens could establish. Additionally, the observation that many of these pathogens actually upregulate adhesion molecule expression as a function of their pathogenesis would indicate a necessity for pathogen-carrying ticks to down regulate this induced expression to facilitate their own feeding purposes.

It is noteworthy, that just as *B. burgdorferi* regulation of adhesion molecule expression may be the result of pathogen interaction with other cells, i.e the ability of *B. burgdorferi* to stimulate macrophage production of IL-1β (Habicht et al., 1985) and TNF-α (Defosse and Johnson, 1992), tick modulation of adhesion molecule expression *in vivo* may also be due to the ability of SGE to suppress macrophage cytokine production, as reported by Ramachandra and Wikel (1992). Because this study utilized a pure population of endothelial cells, it was confirmed that tick salivary gland molecules can directly modulate adhesion molecule expression *in vitro*. Further studies should be carried out to characterize the mechanism and specific salivary gland molecules responsible for modulation of adhesion molecule expression by endothelial cells.

In addition to mediating cellular trafficking to sites of inflamation through cell-cell interactions, adhesion molecules expressed by both endothelial cells and lymphocytes are important in cytokine production. Endothelial cells that are activated by such factors as cytokines (e.g. IL-1β and TNF-α), endotoxin, lipid products, or ligand binding can induce the phosphorylation and degradation of I-κBα, which is bound to NF-κB in the cytoplasm of the cell. The uninhibited NF-κB can migrate to the nucleus and bind specific sites of the promoter regions of target genes. Subsequent regulation of these target genes will result in the synthesis of a number of proteins including cytokines (e.g. IL-1, IL-6, and IL-8), VCAM-1, and ICAM-1 (Krishnaswamy et al., 1999). The ability of both SGE and saliva to modulate adhesion molecule expression by endothelial cells may be indicative the capability to regulate protein production by these cells.

Although the scope of this study did not include the evaluation of the ability of ticks to modulate endothelial cell cytokine production, further studies examining the influence of tick salivary gland-derived molecules and tick feeding on endothelial cell cytokine production should be carried out. One approach to determine if protein synthesis is altered by ticks would be to examine the ability of ticks to regulate NF-κB function, either the protein itself or the inhibitory molecules (e.g. I-κBα) associated with the messenger. Through use of a protein that is known to be immunosuppressive and secreted, such as the one described by Bergman et al. (2000), the ability of ticks to regulate NF-κB function could be measured *in vitro* (Krishnaswamy et al., 1999).

To examine the influence of SGE and saliva from D. *andersoni* on the expression of the lymphocyte integrins LFA-1 and VLA-4, development of a novel assay was

required. Based on the fundamentals of the endothelial cell assay, a whole-cell ELISA was designed to test the ability of tick salivary gland molecules to modulate the constitutive and mitogen-induced expression of lymphocyte integrins. To the best of my knowledge, the ability to upregulate expression of either LFA-1 or VLA-4 by culturing cells with a stimulant has not been reported. In fact, it has been suggested that increased adhesiveness of T lymphocytes is dependent on conformational changes in the integrin that increase affinity for ligands, and not the quantity of surface expression (Springer, 1995). While this appears to be consistent with the finding for VLA-4 in this study, increased expression LFA-1 expression was regularly induced on lymphocytes recovered from BALB/c mice throughout this research. Furthermore, the ability of tick SGE to downregulate this induced expression was also consistently observed.

In some tick-host models repeated exposure to tick feeding results in the development of acquired resistance to ticks. This acquired resistance to feeding is characterized by reduced engorgement weight, diminished ova production, and death of feeding ticks (Wikel, 1996a; 1999). An immunological basis for the expression of acquired resistance has been established, and specific immune responses involved in the manifestation include: antigen-presenting cells, complement-dependent immune effector mechanisms, B and T lymphocytes, antibodies, and cytokines (Wikel, 1996a; 1996d; 1999).

The modulation of lymphocyte integrin expression in tick-naive mice provided the stimulus to investigate the relationship of SGE and saliva to changes induced by tick infestations. BALB/c mice infested with *D. andersoni* have decreased levels of both

constitutive and Con A-induced expression of T lymphocyte LFA-1 and VLA-4, when compared to uninfested controls in both infestations. Furthermore, the extent of down regulation of lymphocyte expression of both LFA-1 and VLA-4 was less after the second infestation, compared to the first infestation. This study reports another pathway by which ticks can modulate host inflammatory and immune responses. Moreover, the decrease in the ability of the tick to suppress the expression of lymphocyte adhesion molecules during repeated infestations is comparable to the pattern of suppression observed for lymphocyte responsiveness to mitogen in another tick-host model, for which acquired resistance has been described (Wikel, 1982).

Acquired resistance was expressed during the second infestation of BALB/c mice with 10 *D. andersoni* nymphs. The observed decreases in both mean engorgement weight and viability of nymphs are consistent with acquired resistance reported for other tick-host relationships (Wikel, 1999). In addition, the results of this study are consistent with the reported development of acquired resistance by BALB/c mice repeatedly infested with *D. variabilis* larvae (denHollander and Allen, 1985a). In a similar manner, guinea pigs infested with *D. andersoni* larvae develop resistance to tick feeding after one infestation (Allen, 1973). The results of this study differed from those reported for BALB/c mice infested with either *I. scapularis* nymphs (Schoeler et al., 1999) or *I. ricinus* nymphs (Mbow et al., 1994a), in that acquired resistance did not develop over the course of repeated infestations.

In addition to down regulation of integrin expression, other T lymphocyte responses were affected by *D. andersoni* infestation. Lymphocyte *in vitro* proliferation,

in response to Con A, was suppressed in mice that were exposed either once or twice to ticks. Similar to the pattern of expression of lymphocyte integrins, upon the second exposure to ticks, the magnitude of suppression was less than that observed after the first infestation. Suppression of T lymphocyte proliferation would result in diminished host cell mediated and antibody immune responses, which would be beneficial to the tick during the days required to obtain a blood meal (Wikel, 1996a).

Suppression of host T lymphocyte responsiveness to mitogens has been reported for a number of different tick-host associations (Wikel, 1982). Lymphocytes collected from guinea pigs infested with *D. andersoni* larvae had significantly suppressed Con A induced proliferation during both first and second exposures (Wikel, 1982). Similar to the results of this study, the magnitude of suppression of Con A-induced *in vitro* proliferation was less intense during the second infestation. Infestation of C3H/HeJ mice with *R. sanguineus* adults led to decreased Con A-induced proliferation of lymph node cells after the first and fourth infestations, which were the only time points assessed (Ferreira and Silva, 1999). Modulation of Con A-induced proliferation has been linked to salivary gland-derived molecules. Normal lymphocyte *in vitro* responsiveness to Con A was suppressed by whole SGE (Ramachandra and Wikel, 1992) and specifically, by a 36 kdal protein in salivary glands of female *D. andersoni* (Bergman et al., 1998).

T and B lymphocyte activation in response to tick antigens has an established role in development and expression of acquired resistance (Wikel, 1996a). In this study, antigen specific *in vitro* lymphocyte blastogenesis, induced by tick salivary gland-derived molecules, was observed following both infestations. These findings are consistent with

an earlier report in which lymphocytes collected from guinea pigs infested with *D. andersoni* larvae proliferated *in vitro* when cultured in the presence of a *D. andersoni* salivary gland antigen (Wikel et al., 1978). Furthermore, the antigen-specific *in vitro* proliferation correlated temporally with the expression of acquired resistance (Wikel et al., 1978). In the present study, the magnitude of SGE-induced *in vitro* lymphocyte proliferation was greater in mice that were exposed twice to ticks. Tick saliva also significantly stimulated increased *in vitro* proliferation of lymphocytes from infested mice, however, an increase in magnitude of proliferation between the first and second infestations was not evident. The magnitude of proliferation induced by SGE was greater than that induced by saliva. Increased lymphocyte stimulation observed with SGE is likely due to a larger number and variety of antigenic molecules present in the salivary gland preparation, when compared to saliva collected only during days four through six of feeding.

T lymphocyte subsets have critical roles as regulators and effectors of host immune responses to ticks (Wikel, 1996a). Differentiation of T lymphocytes into either a Th1 or Th2 phenotype, and the resultant cytokines produced by these subsets, affect the development of host immunity to infectious agents (Mosmann and Coffman, 1989; Fresno et al., 1997), as well as blood-feeding arthropods (Wikel, 1999). In this study, a polarization toward a Th2 cytokine profile after both infestations, with a concomitant decline in production of the Th1 cytokine, IL-2 was observed. Interestingly, infestation resulted in an increase of IFN-γ, another Th1 cytokine, after the first exposure, but did not affect IFN-γ levels after a second infestation. After both infestations, there were

significant increases in the levels of IL-4 and IL-10. Increased production of these cytokines is important in determining the type of immune response that develops, since IL-4 drives differentiation of naive T cells towards a Th2 phenotype (Yaw-Chyn et al., 1999) and IL-10 down-regulates Th1 cytokines (O'Farrell et al., 1998).

Other tick-host associations are characterized by polarization toward Th2 cytokine production. BALB/c and C3H/HeN mice do not develop acquired resistance to *I. scapularis* nymphs; however, infestation results in a down regulation of the Th1 cytokines IL-2 and IFN-γ and an up-regulation of IL-4 and IL-10 (Schoeler et al., 1999). Infestation of BALB/c mice with *I. ricinus* nymphs, a species closely related to *I. scapularis*, result in lymph node lymphocyte production of high levels of IL-4 and low levels of IFN-γ (Ganapamo et al., 1995). Likewise, C3H/HeJ mice repeatedly infested with *R. sanguineus* adults have Th2 polarized cytokine responses (Ferreira and Silva, 1999).

Recently, Ferreira and Silva (1999) suggested that host resistance or susceptibility to tick infestation is likely determined by tick modulation of host cytokines. The contribution of individual cytokines to development and expression of acquired resistance remains unknown. It is however interesting to speculate on the significance of IFN-γ in the development of acquired resistance to tick feeding. As previously discussed, BALB/c mice infested with either *I. scapularis* or *I. ricinus* do not develop acquired resistance to ticks, therefore, it is interesting to note the differences in cytokine production between those tick-host models and the one used in this study.

In both of the studies utilizing *Ixodes*-BALB/c models, there was significant suppression of IFN-γ during successive infestations, however in this study, after two infestations, the level of IFN-γ produced by Con A-stimulated T lymphocytes from mice infested with ticks did not significantly differ from the levels observed for control mice. Although there is clearly an enhanced shift in the cytokine production towards a Th2 profile, as evidenced by significant decrease in IL-2 and significant enhancement of IL-4 and IL-10 from the first to the second infestation, the presence of IFN-γ may be involved in the redifferentiation of Th2 cell to a Th1 phenotype. Recently, it has been shown that Th1 cells are capable of transducing IL-4 signals, and can be redifferentiated toward a Th2 phenotype by secondary antigen stimulation in the presence of IL-4. In contrast, Th2 cells are refractory to IL-12-driven redifferentiation toward a Th1 phenotype due to downregulation of the β_2 chain of the IL-12 receptor during initial Th2 differentiation. However, in the presence of IFN-γ, expression of the β_2 chain of the IL-12 receptor is restored, and cells can be redifferentiated into Th1 cells (Lim et al., 1999).

Additional evidence implicating the influence of IFN-γ production to the development of acquired resistance involves the activation of endothelial cells. Selective transmigration of Th1 cells, but not Th2 cells, across IFN-γ-stimulated endothelial cells *in vitro* has recently been reported (Kawai et al., 1999). Combined with the ability of IFN-γ to influence redifferentiation of Th2 cells, this selective transmigration of Th1 may provide a basis for the cellular infiltration observed in tick-resistant hosts.

The interaction of adhesion molecules can also be important in the differentiation of T helper lymphocytes. By blocking the binding of LFA-1 on naive $CD4^+$ T cells to

ICAM-1 expressed by activated dendritic cells, the characteristic ability of dendritic cells to induce a Th1 type response is not only inhibited, but there is also a significant shift towards Th2 cytokine production (Salomon and Bluestone, 1998). The influence of tick feeding on the interactions of LFA-1 expressed on lymphocytes and ICAM-1 on dendritic cells has yet to be examined. However, based on the observations of this study it seems plausible that ticks could modulate ICAM-1 expression on dendritic cells, contributing to the polarization towards a Th2 cytokine profile.

The ability of ticks to suppress host immune responses, including complement activation, antibody production, and lymphocyte function, as well as macrophage and T cell cytokine production is necessary to successfully obtain a blood-meal. Molecules present in the saliva of ticks also facilitate feeding by inhibiting host blood coagulation, prevent platelet aggregation, pain, increasing vasodilation, and modulating host immunity (Ribeiro, 1995; Wikel and Bergman, 1997; Wikel, 1999). It is becoming increasingly apparent that tick modulation of the host immune response is important for pathogen transmission (Wikel, 1999). Although pathogens have developed their own mechanisms of immune subversion (Marrack and Kappler, 1994), it is of obvious benefit to the pathogen to engage a new host during a period of time, and at a location, in which the immune response is suppressed.

The results of this research suggest a new aspect of how tick salivary gland molecules might influence important aspects of host inflammatory/immune defenses. Both *D. andersoni* SGE and saliva down regulate the constitutive and inflammatory stimulus-induced expression of E-selectin, VCAM-1 and ICAM-1 by murine endothelial

cells, as well as the Con A-induced expression of LFA-1 and VLA-4 on lymphocytes from tick-naive mice *in vitro*. Through identification of the molecule(s) in salivary glands and saliva responsible for the suppression of adhesion molecules one could target either the molecule itself, or its mode of action, and essentially block the tick's ability to suppress host defenses (Wikel, 1999).

The findings of this study suggest a balance between host acquired immunity to infestation and the ability of the tick to down-regulate or polarize host immune responses in a manner that facilitates blood-meal acquisition. Additionally, differences in the immune response of a host that develops acquired resistance, compared to those tick-host associations in which acquired resistance does not develop, have been identified. Further studies should examine the role of individual cytokines in the development of acquired resistance. By use of cytokine-specific knockout mice (e.g. IFN-γ) the contribution of each cytokine to the development of acquired resistance could be assessed.

The endothelial cell research should be expanded in two different ways. First, using the system developed for this study, the influence of tick salivary gland-derived molecules on the influence of endothelial cytokine production should be determined. Secondly, immunostaining techniques similar to those describe by Mbow et al. (1994b) should be used to identify expression patterns of the adhesion molecules described in this study, with the tick-host model also used in this research. Differences in adhesion molecule expression in a tick-host model in which acquired resistance has been described could provide insight into the role of adhesion molecules in the expression of acquired resistance.

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APPENDICES

APPENDIX I

Reagent Compositions

Section Page

Section 1: Buffers and Media Compositions

Phosphate Buffered Saline (PBS, 0.15 M, pH 7.2)

1. Dissolve the following in 900 ml of triple distilled water:

Potassium phosphate (monobasic, KH_2PO_4)	2.45 g
Sodium phosphate (dibasic, Na_2HPO_4)	8.10 g
Sodium chloride (NaCl)	4.38 g

2. Bring final volume to one liter. The pH does not require adjustment.

Dulbecco's Modified Eagle Medium (DMEM)

1. To make a 1 X concentration, use one package of powdered DMEM (Gibco BRL, Cat # 12100-046).
2. Measure out 950 ml of triple distilled water into a flask that is as close to the final volume (1 liter) as possible.
3. Add the contents of the package to water with gentle stirring, at RT.
4. Rinse out all traces of powder from package, add to flask.
5. Add 3.7 g of Sodium bicarbonate ($NaHCO_3$) per liter to the solution.
6. Bring final volume to 1 liter.
7. Stir until dissolved.
8. Adjust the pH to 0.2-0.3 below desired pH (example: 6.9 for final pH of 7.2), with 1N NaOH or 1N HCl.
9. Filter sterilize immediately using 0.22 μm nitrocellulose membrane sterivex filter unit with bell fitting (Millipore).
10. Incubate 10 ml of sterile medium at 37°C overnight for sterility check.
11. Store remaining medium in a sterile 1 liter media bottles (Corning) at 4°C.

RPMI 1640 Medium

1. To make a 1 X concentration, use one package of powdered RPMI 1640 (Gibco BRL, Cat # 31800-022).
2. Measure out 950 ml of triple distilled water into a flask that is as close to the final volume (1 liter) as possible.
3. Add the contents of the package to water with gentle stirring, at RT.
4. Rinse out all traces of powder from package, add to flask.
5. Add 2.0 g of Sodium bicarbonate ($NaHCO_3$) per liter to the solution.
6. Bring final volume to 1 liter.
7. Stir until dissolved.
8. Adjust the pH to 0.2-0.3 below desired pH (example: 7.1 for final pH of 7.4), with 1N NaOH or 1N HCl.

9. Filter sterilize immediately using 0.22 μm nitrocellulose membrane sterivex filter unit with bell fitting (Millipore).
10. Incubate 10 ml of sterile medium at 37°C overnight for sterility check.
11. Store remaining medium in a sterile 1 liter media bottles (Corning) at 4°C.

Section 2: ELISA Buffers and Reagents

Whole-cell ELISA

Wash buffer (PBS with 1 mM $CaCl_2$, 1 mM $MgCl_2$)

1. Dissolve 0.147 g $CaCl_2$ and 0.203 g $MgCl_2$ in 1 liter of PBS.
2. Store buffer in a sterile 1 liter media bottle (Corning) at 4°C.

Assay diluent (1% Bovine serum albumin (BSA) in PBS)

1. Dissolve 1.0 g of BSA in 100 ml of PBS.
2. Store solution in a glass bottle at 4°C. Make fresh solution for each assay. Do not store for more than five days.

Fixing solution (4% Paraformaldehyde in PBS)

1. Dissolve 4.0 g of paraformaldehyde in 100 ml of PBS. Requires gentle heating to dissolve paraformaldehyde.
2. Let solution cool to RT, and store in glass bottle at RT.

Substrate (OPD; 300μg/ml in 50 mM K_2HPO_4; 25 mM Citric acid)

1. Dissolve 8.709 g K_2HPO_4 and 5.25 g Citric acid in 1 liter of triple distilled water.
2. Adjust the pH to 6.0 with 1N NaOH.
3. This will be buffer solution. Store buffer in a sterile 1 liter media bottle at 4°C.
4. To make OPD for assay, dissolve one 10 mg tablet of OPD (Sigma) to 33.3 ml of prepared buffer.
5. Just before use in ELISA, add 7 μl of 30% H_2O_2 to the 33.3 ml of OPD solution.

Stop solution (HCl)

1. Add 8.3 ml of stock 1 M HCl to 91.7 ml of triple distilled water.
2. Store stop solution in glass bottle at RT.
3. Addition of 50 μl the stop solution to 100 μl of substrate already present in the well will provide a 1 mM final concentration in the well.

Antigen-capture ELISA

Coating buffer (0.1 M Carbonate, pH 9.5)

1. Dissolve 8.40 g $NaHCO_3$ and 3.56 g Na_2CO_3 in 1 liter of triple distilled water.
2. Adjust the pH to 9.5 with 1N NaOH or 1N HCl.
3. Store buffer in a sterile 1 liter media bottle at 4°C, for no more than 30 days.

Wash buffer (PBS with 0.05% Tween-20)

1. Dissolve 500 μl of Tween-20 in 1 liter of PBS.
2. Store buffer in a sterile 1 liter media bottle at 4°C, for no more than 30 days.

Assay diluent (10% Fetal bovine serum (FBS) in PBS)

1. Add 10.0 ml of heat-inactivated FBS (HyClone) to 90 ml of PBS.
2. Adjust the pH to 7.0 with 1N NaOH or 1N HCl.
3. Store solution in a glass bottle at 4°C. Make fresh solution for each assay. Do not store for more than five days.

Substrate (Tetramethylbenzidine (TMB) and H_2O_2)

1. Bring both solutions to RT, but do not heat or use water bath.
2. Just prior to use, mix equal volumes of TMB and H_2O_2 into a test tube.
3. Use appropriate amount for ELISA, and discard remaining mixture.

Stop solution (2N H_2SO_4)

1. Add 5.55 ml of stock H_2SO_4 to 99.45 ml of triple distilled water.
2. Store stop solution in glass bottle at RT.

APPENDIX II

In vitro splenocyte proliferation data presented as actual CPM ± S.E.M. obtained in response to the mitogen Con A, and tick salivary gland antigen, for BALB/c mice repeatedly infested with *D. andersoni* nymphs.

Table A. *In vitro* proliferative responses of splenocytes from BALB/c mice, infested one or two times with 10 *D. andersoni* nymphs, to Concanavalin A (Con A).

Infestation/ Group	Treatment			
	0.0 μg Con A	0.5 μg Con A	1.0 μg Con A	2.0 μg Con A
First Infestation				
Control	972 ± 104	183016 ± 15959	177925 ± 15107	152082 ± 12465
Infested	1387 ± 138	192981 ± 16564	183718 ± 18881	147339 ± 12156
Second Infestation				
Control	902 ± 44	200245 ± 8505	195659 ± 7948	176908 ± 6519
Infested	1299 ± 128	223569 ± 7444	210155 ± 8735	178324 ± 7548

5×10^5 splenocytes from infested and control mice were incubated with varying concentrations of Con A for 54 hours and then pulsed with one μCi of ^{3}H-thymidine for 17 hours. Cells were harvested and radioactivity incorporation was determined by counting in liquid scintillation counter. Data are presented as counts per minute (±S.E.M.).

Table B. *In vitro* proliferative responses of splenocytes from BALB/c mice, infested one or two times with 10 *D. andersoni* nymphs, to salivary gland extract (SGE).

Infestation/ Group	Treatment			
	0.0 μg SGE	0.1 μg SGE	0.5 μg SGE	1.0 μg SGE
First Infestation				
Control	1170 ± 111	1040 ± 49	1020 ± 52	1116 ± 62
Infested	1983 ± 63	4949 ± 656	9218 ± 735	12339 ± 914
Second Infestation				
Control	774 ± 42	683 ± 29	713 ± 40	691 ± 31
Infested	1242 ± 109	2675 ± 298	6719 ± 715	8326 ± 728

5×10^5 splenocytes from infested and control mice were incubated with varying concentrations of SGE for 54 hours and then pulsed with one μCi of ^{3}H-thymidine for 17 hours. Cells were harvested and radioactivity incorporation was determined by counting in liquid scintillation counter. Data are presented as counts per minute (± S.E.M.).

Table C. ***In vitro*** **proliferative responses of splenocytes from BALB/c mice, infested one or two times with 10** ***D. andersoni*** **nymphs, to saliva.**

Infestation/ Group	Treatment			
	0.0 μg saliva	0.1 μg saliva	0.5 μg saliva	1.0 μg saliva
First Infestation				
Control	1599 ± 217	1110 ± 81	1378 ± 109	1254 ± 135
Infested	2103 ± 118	2353 ± 182	4769 ± 93	5988 ± 288
Second Infestation				
Control	526 ± 30	484 ± 21	462 ± 31	452 ± 5
Infested	1736 ± 419	2170 ± 517	4661 ± 1344	5189 ± 1338

5×10^5 splenocytes from infested and control mice were incubated with varying concentrations of saliva for 54 hours and then pulsed with one μCi of ^{3}H-thymidine for 17 hours. Cells were harvested and radioactivity incorporation was determined by counting in liquid scintillation counter. Data is presented as counts per minute (± S.E.M.).

VITA

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Doctor of Philosophy

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