IN VITRO CYTOTOXIC ACTIVITY OF EQUINE LYMPHOCYTES ON EQUINE HERPESVIRUS-1 INFECTED ALLOGENIC FIBROBLASTS

by

Lucy Marie Edens, DVM

Thesis submitted to the Faculty of the

Virginia-Maryland Regional College of Veterinary Medicine

in partial fulfillment of the requirements for the degree of

MASTERS OF SCIENCE

in

Veterinary Medical Sciences

APPROVED:

M. J. Murray, Chairman

M. V. Crisman

T. E. Toth

S. A. Ahmed

May, 1993

Blacksburg, Virginia

Dedicated to my husband

Lee A. Young, D.V.M.

for his support and understanding.

IN VITRO CYTOTOXIC ACTIVITY OF EQUINE LYMPHOCYTES ON EQUINE HERPESVIRUS-1 INFECTED ALLOGENIC FIBROBLASTS

by Lucy Marie Edens

Committee Chairman: Michael J. Murray
Veterinary Medical Sciences

(ABSTRACT)

The objectives of this study were to: 1) develop a technique to analyze the in vitro cytotoxic activity of lymphocytes from adult horses against equine herpesvirus-1 (EHV-1) infected allogenic equine dermal fibroblasts (EDF); 2) evaluate the ability of a 72 hour in vitro incubation with interleukin-2 (IL-2) to enhance the lymphocytic cytolytic activity against EHV-1 infected EDF; 3) compare the cytotoxic activity among lymphocytes isolated from pregnant mares and non-pregnant mares against EHV-1 infected EDF; 4) ascertain if any correlations existed between the percent cytotoxicity and percentage of lymphocytes phenotypically identified by five different mouse-anti-equine monoclonal antibodies; and 5) determine if any correlation existed between virus-neutralizing antibody titers and the percent cytotoxicity.

Results of the study indicate that in vitro cytotoxic activity of equine lymphocytes

against EHV-1 infected allogenic fibroblasts can be measured with a standard 4 hour ^{51}Cr release assay. This activity was enhanced by an in vitro incubation with IL-2. The cytolytic activity of freshly isolated lymphocytes was greater for non-pregnant than pregnant mares. However, after IL-2 stimulation the cytolytic activity was greater for lymphocytes from pregnant mares. A positive correlation was not detected between the percentage of phenotypically identified cells and the percent cytoxicity, although several negative correlations were present. This suggests that the cytotoxic activity was either not mediated by any of the phenotypically identified cell populations or that the activity was mediated by several different cell populations. No correlation was detected between virus-neutralizing antibody titers and the percent cytotoxicity.

ACKNOWLEDGEMENTS

The author would like to thank the Paul Mellon Equine Research Foundation for providing the majority of the financial support for this project. Additional contributions were obtained from the USDA - animal health and disease, and the Hills student research fund.

The author would also like to thank Dr. John Ortaldo, National Cancer Institute, Frederick Maryland, for the generous contribution of his time and expertise, laboratory facilities, and technical assistance; Dr. George Allen for supplying the equine herpesvirus-1 (Kentucky D strain) and the anti-glycoprotein-14 antibody; Dr. William Davis for his contribution of the mouse-anti-equine monoclonal antibodies and helpful advice.

I am very grateful to all the members of my committee including Dr. Murray for providing me with this opportunity; Dr. Toth for his advice and support; Dr. Ahmed for his patience and guidance; and Dr. Crisman for stimulating my interest in immunology.

Although there are many other people whom I would like to express my appreciation to, those who must be mentioned include:

My friends and fellow graduate students who made the many long hours in the laboratory and time I spent in Blacksburg fun.

Megan Irby, Haywood Pyle, Cathy Rogers, and Stephanie Shane for their outstanding technical assistance.

Pat and Pam Ray for their friendship, guidance, and home cooked meals.

and Julie Webb for her unrelenting encouragement and support.

IN VITRO CYTOTOXIC ACTIVITY OF EQUINE LYMPHOCYTES ON EQUINE HERPESVIRUS-1 INFECTED ALLOGENIC FIBROBLASTS

TABLE OF CONTENTS

GENERAL INTRODUCTION

Equine herpesvirus-1 (EHV-1) is a ubiquitous pathogen of horses which causes considerable economic loss to the horse industry as a consequence of respiratory, neurologic, and abortifacient symptoms.[1,2] Infection is acquired through inhalation of infective virus particles with subsequent viral replication in respiratory epithelial and endothelial cells.[3] Dissemination of the virus occurs as a result of a cell-associated viremia in lymphocytes and monocytes, producing widespread endothelial cell infection.[4] Although horses of all ages are vulnerable to EHV-1 infection, young, immunologically naive horses and pregnant mares are the groups most commonly and severely affected.[1] The increased risk of clinical disease associated with pregnancy increases as the duration of pregnancy increases, culminating frequently in late gestational abortion or paresis.[5,6,7,8,9] Infection of the respiratory tract and the ensuing viremia occurs despite high levels of virus-neutralizing antibody.[10,11] This implies that cellular responses, particularly cytotoxicity, is the most important aspect of immunity necessary to limit viral replication and spread of EHV-1.

Cellular cytotoxicity mediated through specific and non-specific immunologic responses is known to be the crucial factor for immunocompetence against disseminated herpesviral infections in other species.[12,13,14,15]

Investigations of herpesviral infections in laboratory animals, cattle, and human beings have demonstrated increased morbidity and mortality associated with down-regulation of cellular cytotoxic responses.[16,17] One of the most striking examples of this phenomenon is the pregnancy-related susceptibility of women to cytomegalovirus and herpes simplex virus infection or reactivation of latent infections. In this situation, immunosuppression increases with advancing duration of pregnancy.[18,19,20,21] Cytotoxic responses return to normal shortly after parturition, often coinciding with resolution of the clinical manifestations of infection.[22,23]

Cell-mediated immunity to EHV-1 infection has been analyzed primarily by lymphocyte proliferation in response to heat inactivated viral antigens. These assays provide evidence of T lymphocyte recognition and response to viral antigens, but do not reflect cytotoxic activity against viral infection.[24,25,26] Limited evaluation of cellular cytotoxicity in horses has yielded conflicting information. Magnuson, et al. were unable to demonstrate non-specific cytotoxic responses from adult horses to a murine tumor cell (YAC-1).[27] In vitro stimulation with human recombinant IL-2 failed to induce cytotoxic activity in this model, although antibody-dependent cellular cytotoxicity (ADCC) was present. In contrast, non-specific cell-mediated cytolysis of an equine tumor cell line, EqT8888, was detected after a 72 hour in vitro incubation of equine lymphocytes with IL-2.[28] Investigation into immunity to EHV-1 infection has identified ADCC against virus

infected target cells by neutrophils isolated from young horses after experimental infection with EHV-1.[29,30] Chong et al. identified spontaneous cytotoxic activity against EHV-1 infected fibroblasts from lymphocytes isolated from 1 adult horse, although a thorough investigation was not conducted.[31]

The purpose of this study was to develop an in vitro technique to evaluate the cytotoxic activity of non-adherent equine peripheral blood mononuclear cells (lymphocytes) against EHV-1 infected target cells. This technique was employed to compare cytotoxic activity of lymphocytes from pregnant and non-pregnant mares on EHV-1 infected cells. Concurrent identification of isolated mononuclear cell populations by immunophenotypical analysis was used to evaluate changes in lymphocyte subsets.

I. OVERVIEW OF THE IMMUNE RESPONSE TO VIRAL INFECTION

A. Introduction

The ability of horses to resist invasion and infection by viral pathogens is dependent on the competency of specific and non-specific immunologic responses. Innate immunity is the inherent, non-specific resistance to infection which arises in the absence of previous exposure to the offending pathogen. Because protection is provided without prior stimulation it is non-specific, unable to distinguish between different antigens. This precludes any alteration in the response upon repeated exposure to an identical virus. This differs from acquired immunity, where a specific response develops for each immunogenic viral antigen. The specificity of the reaction dictates that a period of time must lapse between initial viral invasion and host response. The benefit of acquired immunity is the rapid generation of an antigen-specific immune response upon subsequent exposure to the antigen.

Given this disparity between the two immune responses, the question might arise if both types of immunity are actually necessary for the body to defend itself against viral infections. Consider the extent of damage which could result from a viral infection in an immunologically naive host, if the infection progressed unrestricted for 5 to 7 days, until a specific immune response developed. However, some viruses possess the capacity to evade the non-specific immune response necessitating a specific response to eliminate them. The absence of

immunologic memory would leave the body continually at risk of infection. Therefore, a deficiency in either the innate or acquired immune response increases the morbidity and mortality rates associated with virus-induced diseases.

The purpose of this segment of the literature review is to familiarize the reader with the components of the innate and acquired immune responses. Examples of specific viral diseases will be used to illustrate the clinical relevance of these points in equine medicine.

B. Innate Immunity

1. Introduction and Overview

The protection provided by the innate immune system consists of genetic restriction, external barriers, physiologic defenses, humoral factors, and a variety of cellular mechanisms. Genetic restriction results from an incompatibility between the host cells and viral pathogens on a molecular level. For instance, asinine herpesvirus-3 (AHV-3), a pathogen antigenically similar to EHV-1, is unable to infect horses because of differences in DNA between the host species.[32] The external barriers to the entry of viral pathogens consist predominantly of epithelial surfaces such as the skin, cornea, intestinal tract, and respiratory system which physically impede the penetration of pathogens.[33] Mucus lining the respiratory and gastrointestinal tract can entrap viruses, preventing attachment and enhancing clearance.[34,35] Secretory products, which include lacrimal lysozyme,

gastric acid, bile acids, and pancreatic and intestinal proteases contribute to antiviral defenses by either altering the microenvironment to prevent adhesion or producing irreparable damage to the virus.[36] While these genetic, physical, and physiological factors act to reduce viral entry, they are not impervious. The underlying tissues are continuously challenged with pathogenic viruses which have avoided these defense mechanisms.

2. Humoral Factors

The circulating proteins of the complement system are naturally present in normal horses and conduct a wide variety of antiviral functions.[37,38] Two distinct routes of complement activation exist. The classical pathway is activated by the binding of immune complexes, containing IgM or IgG to the complement protein C1, requiring previous exposure to the virus and the production of virus-specific, complement-fixing antibodies.[39] However, activation of complement can occur in the absence of antibodies, representing a type of non-specific humoral defense mechanism.[40,41] C3 can be activated through the alternative pathway by direct viral attachment to C3 in the absence of antibodies.[42] In horses EHV-1 induces the expression of complement receptors on infected cells, through which antibody-independent complement-mediated cytolysis is generated.[30,43]

3. Cellular Mechanisms

The effector cell population constituting the cellular component of the innate immune response is composed of three classes of cells, polymorphonuclear

leukocytes (PMN), macrophages, and natural effector cells. The primary anti-viral protective properties of macrophages and PMN's occur in association with the acquired immune response and are discussed under the acquired immune response.

Natural effector cells (NEC) are lymphocytes with the ability to mediate a variety of immunologic responses in the absence of prior sensitization.[44] The three cell types classified in this group are natural killers cells, natural cytotoxic cells, and lymphokine activated killer cells. These cells were originally identified by their spontaneous cytolytic activity against neoplastic cells.[45] Later, it was discovered that NEC had in vitro cytotoxic activity against a myriad of different viruses.[46] The role of NEC in controlling viral infections appears to be particularly important in limiting initial viral replication.[47] The spectrum of antiviral effects exhibited by NEC includes activity against a number of different viral pathogens, most notably herpesviruses and influenza.[48,49,50] The in vivo significance of the antiviral activity of NEC has been highlighted by several recent reports of severe recurrent viral infections in humans deficient in natural cytotoxic activity.[51,52,53] Spontaneous cytotoxic activity against EHV-1 infected cells has been detected in immunologically naive, specific pathogen free ponies.[54]

Although functionally and phenotypically diverse, morphologically NEC appear as large granular lymphocytes.[55] Purified preparations of equine large granular lymphocytes isolated by density-gradient separation have cytotoxic

activity against neoplastic cells and virus infected cells in vitro chromium release assays.[27,56] Large granular lymphocytes comprise approximately 5% of peripheral blood lymphocytes and 3% of the total circulating mononuclear cell population.[57,58,59] They lack most of the characteristics associated with macrophages, PMNs, or T lymphocytes.[60] The characteristics which distinguish NEC from macrophages are their inability to adhere to glass or plastic, lack of phagocytic activity, absence of intracellular esterase-containing granules, and the presence of surfaces receptors for IgG.[61] PMN phagocytic activity and direct cytolytic ability differentiates NEC from PMN.[62] The separation between NEC and T lymphocytes is based on the absence of a T cell receptor (TCR) and TCR genes.[63]

Natural effector cells function without MHC-restriction, thus lysing allogenic as well as autologous infected cells.[64,65] This is in contrast to cytotoxic T lymphocytes whose anti-viral functions are predominately regulated through the recognition of self-MHC molecules in combination with viral antigens. The cytotoxic activity of both NEC and cytotoxic T lymphocytes is modulated by interferon and interleukin-2.[28,66] The participation of these cytokines in augmenting the in vivo immune response is particularly important in the context of anti-viral immunity. Interferon concentrations increase at the site of infection and systemically in horses during the initial stages of viral infection resulting in enhanced activity of NEC.[67,68,69,70] Equine lymphocytes demonstrate

increased cytolytic activity against virus infected cells after in vitro incubation with interleukin-2.[71] Equine NEC appear to play an important role in the horses ability to defend itself against viral infections.

C. Acquired Immunity

1. Introduction

Acquired antiviral immunity results from a series of integrated interactions between lymphocytes, antigen presenting cells, and viral antigens. The end products, antibodies and cytotoxic T lymphocytes, are produced as a consequence of genetically restricted interactions between the cellular constituents. All nucleated cells in the body contain surface molecules, major histocompatibility complexes (MHC), which regulate immune-mediated intercellular communication.[72] The species specific designation of the MHC assigned to horses is the Equine Leukocyte Antigen (ELA), with different classes of ELA antigens, class I and class II, identified.[73,74] Preliminary characterization of ELA-interactions indicates functional homology between ELA classes I and II, and class I and class II MHC-restricted interactions of other species.[75,76,77,78] In addition to MHC-restriction, the antigenic characteristics of virus and the method of presentation to the immune system dictate the type of response generated. In most situations, the entire spectrum of responses results from viral infection, however for different viruses, different responses predominate. Horses anti-viral protective capability is dependent on appropriate function of this intricate

communication system.

T lymphocytes are divided into two distinct groups based on their MHC class restriction. Class I restricted T lymphocytes, designated EqCD8, are commonly referred to as cytotoxic T cells.[79,80] T lymphocytes functioning in a class II restricted manner, called T helper cells, are denoted as EqCD4.[74] Phenotypic identification of T lymphocytes is determined by labeling with monoclonal antibodies directed toward structures located on the cell membrane surface. Functional properties are associated with these different lymphocyte antigens and are evaluated by blocking the surface structure with its identifying monoclonal antibody. Whereas, the MHC class restriction affiliated with different lymphocytic surface structures is homogenous, the functional characteristics of these cells are not as discrete. However, several functional traits are frequently assigned to these lymphocytic subpopulations. The majority of T lymphocytes whose activity is class I MHC restricted, EqCD8, operate as cytotoxic cells.[81,82,83,84,85] Class II MHC restricted T lymphocytes, EqCD4, are specified as T helper cells, participating indirectly through antigen presentation and cytokine secretion.[82]

2. Antibodies

Antibodies are complex, globular proteins produced by plasma cells which specifically react with the antigen that induced their production.[86] Although the production of antibodies is considered to be a function of the humoral immune

response, a complex series of events combining antigen presenting cells, T helper cells (Th), cytokines, and B lymphocytes is necessary before the end result, an antibody producing plasma cell, is generated. The first step in antibody production is antigen processing and expression by antigen presenting cells such as macrophages.[87] The processed antigen is then displayed on the surface of the antigen presenting cell in combination with class II histocompatibility molecules.[88] The antigen-class II complex is recognized by T helper cells through the cell membrane surface molecule EqCD4.[89] Activated T helper cells recognize and stimulate B cells recognizing the same antigen, again in a class II restricted manner.[90] Interleukins 4 and 5 secreted by T helper cells, act synergistically to promote B lymphocyte proliferation and induce their differentiation into plasma cells. [91,92] Interleukin-6 magnifies the secretion of antibodies.[93]

Antibodies protect against viral infection through a number of different mechanisms. These protective functions include direct inactivation, promotion of complement-dependent lysis, enhancement of phagocytosis, and antibody-dependent cellular cytotoxicity. Virus neutralizing antibodies are highly specific, but only effective against extracellular viruses. Viruses such as EHV-1 which are disseminated through a cell-associated viremia and direct cell to cell contact, are not effectively neutralized by antibodies.

Horses have a well developed mucosal immune system where the virus-neutralizing effects of antibodies are probably most important in viral

immunity.[94,95,96] Secretory IgA is particularly effective against viruses, such as equine influenza, EHV-4 and equine rhinovirus, where the primary site of replication is the respiratory epithelium.[97] For adequate mucosal IgA to be produced, either local immunization or recovery from natural infection is necessary.[98,99] Failure of conventional, parenterally administered equine influenza vaccines to stimulate protective immunity of any significant duration may, in part, be related to their inability to induce local immunity.[97] Preliminary investigations with temperature sensitive mutants of equine influenza virus incite local immunity for at least four weeks after intranasal vaccination.[100,101]

Virions or virus infected cells coated with complement-fixing antibodies, of the IgM or IgG isotypes, are subject to lysis through either the classical or alternative complement pathway. The role of antibody-dependent complement mediated cytolysis is important in limiting viral infection of intracellular viruses which express immunogenic viral glycoproteins on the outer cell membrane surface.[102,103] Complement-fixing antibodies do not prevent infection with intracellular viruses such as EHV-1, however they do provide an effective mechanism for the eventual clearance of virus infected cells.[30,43,104,105] Although the incidence of EHV-1 and EHV-4 infections are not reduced by the presence of complement-fixing antibodies, the severity of complications including respiratory disease, abortion, and the duration of viral shedding, are significantly reduced.[21]

Opsonophagocytosis, an important antibacterial defense mechanism, is of relatively minor importance in antiviral defenses. However, certain viruses coated with antibody and complement, are susceptible to phagocytosis by neutrophils and macrophages.[106] Enteric viruses are particularly susceptible to antibody-mediated phagocytosis.[107] The ability of viruses, most notably equine herpesviruses, to replicate within macrophages reflects the virus's capacity to evade enzymatic degradation and may serve as a mechanism through which viral persistence can occur.[108,109] Antibody coating of virions facilitates their entrance into macrophages via antibody receptors, permitting evasion of humoral host defense mechanisms.[110] In certain situations the presence of circulating virus-neutralizing antibodies may contribute to latent or persistent viral infections.

The most important function of antibodies in the resistance to viral infection is mediated through a recently elucidated cellular mechanism, antibody-dependent cellular cytotoxicity (ADCC). In ADCC, a virus infected cell is coated with virus-specific antibodies, usually of the IgG isotype, allowing adherence of Fc receptor-positive leukocytes.[111,112] Antibody-dependent cellular cytotoxicity endows unsensitized cells with virus-specific cytolytic activity. Cells capable of mediating ADCC are subpopulations of PMN, macrophages, and natural killer cells which bear Fc receptors for immunoglobulins.[113,114,115,116] There are species specific disparities in the predominant effector cell mediating ADCC. The primary effector cell is macrophages in mice, natural killer cells in humans, and

neutrophils in horses and cattle.[30,117,118,119] The amount of antibody necessary to mediate this function is small and the cytolytic reaction occurs rapidly, within 2 to 3 hours.[120,121] The proposed in vivo significance of ADCC is in limiting the dissemination of virus infected cells prior to the release of newly synthesized virions. This is important in the initial stages of viral replication when antibody concentrations are low.[122] Antibody dependent cellular cytotoxicity is an effective in vitro mechanism against EHV-1 and EHV-4 infected cells.[30] This response to EHV infection in vitro, is enhanced by vaccination with a killed virus EHV-1 vaccine. However, it is not effective in preventing herpesviral infection in horses experimentally challenged with EHV-1 or EHV-4.[30,123] Therefore, ADCC appears to reduce the severity and extent of equine herpesviral infections, but is not protective against infection.

3. Cytotoxic T Lymphocytes

The primary function of cytotoxic T lymphocytes (CTL) is direct cytolysis of cells presenting viral antigens in association with MHC molecules.[124] Because of the requirement for MHC recognition in conjunction with recognition of viral antigens, CTL interact with virus infected cells but not free virions.[125] The role of CTL in limiting viral infections is especially important in diseases such as equine herpesviruses which spreads by direct intercellular passage rather than by the extracellular route.[126] The cytolysis of virus infected cells is particularly beneficial if the killing occurs before viral replication and dissemination.[127]

Experimental studies using laboratory animals have demonstrated that class I restricted CTL are imperative for recovery from high-dose challenges of herpes simplex virus.[128] A small percentage of T helper cells operate as cytolytic cells recognizing viral antigens in association with MHC class II molecules, contributing to resistance to low dose challenges of herpes virus.[85,129] Recognition of viral antigens by the T cell receptor results in activation of the CTL, expression of surface receptors for IL-1 and IL-2, and finally proliferation and differentiation into lytically active effector CTL.[130,131]

D. Processing of Viral Antigens

The recognition of viral antigens necessary to generate an acquired immune response is mediated through either the T cell receptor or virus-specific antibodies. Antigen binding sites are located within a cleft formed between two polypeptide chains with areas of diversity, known as hypervariable regions, providing sites of attachment unique for each antigen.[132,133] The one fundamental difference is that immunoglobulins react directly with infective virus particles or viral antigens while the T cell receptor only recognizes processed antigens presented on the surface of cells. The method of antigen processing is based on the biochemical and reproductive characteristics of the virus and is the principal determinant of the nature of response generated.[26] Large protein antigens gain entry to the antigen presenting cell through phagocytosis or endocytosis, undergo proteolytic cleavage into small peptide fragments, and are

presented in association with class II MHC molecules on the cell membrane.[134] Antigens which are processed in the cytoplasm rather than within endosomes, are presented in association with class I MHC molecules.[135] A CTL response is not stimulated by inactivated viral antigens.[136] Variability in the immune response resulting from differences in antigen processing has important implications for development of effective forms of immunoprophylaxis.

E. Summary

The body's ability to protect against invasion and infection by pathogenic viruses is essential for its survival. However, as our ability to detect viral infections becomes increasingly sophisticated, it is evident that the host is not always successful in its' attempt to avert infection. Viruses can evade detection by altering the immune response, changing their antigenic characteristics, or by going into hiding (latency) to ensure their persistence. Successful prevention and control of viral diseases is dependent on understanding the immune response to viral infection, evasion of the immune response by viruses, and immunomodulation of the hosts response.

II. EQUINE HERPESVIRUS

A. Introduction

The extent of economic losses to the horse industry as a result of infection with equine herpesviruses has not been specifically calculated because of the universal, ubiquitous nature of the viruses and the absence of adequate

documentation in many suspected disease outbreaks. However, the multitude of epizootics delineated in the scientific literature, occurring on every continent where large populations of horses are located, underscore the potential magnitude of this problem.[137] The prevalence of infection, morbidity rate, and overall mortality vary among the different classes of equine herpesvirus.

1. Classification

Equine herpesviruses are members of the family Herpesviridiae. The structure of the virion of these double-stranded DNA viruses is common to all members, independent of species predilection.[138] There are three subfamilies of herpesviruses - Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae with distinct biological properties.[139] Alphaherpesviruses are identified by their rapid replication, cytolysis of infected cells, and ability to infect a wide range of hosts. Slow growing viruses with a high degree of host specificity are designated as Betaherpesvirinae. The most diverse subfamily of herpesviruses is the Gammaherpesvirinae with variable lengths of reproductive cycles, discrete host specificity, T cell or B cell lymphotropism, high incidence of oncogenicity, and occasional in vitro cytolytic activity.

Herpesviral infections in horses have been attributed to at least four or five distinctly different viruses.[140] Three different equine herpesviruses, equine herpesvirus-1, 3, and 4 (EHV-1, EHV-3, EHV-4), are members of the subfamily Alphaherpesvirinae. The complex differentiation between EHV-1 and EHV-4 will

be discussed in the section below. The clinical syndrome resulting from infection with EHV-3, known as equine coital exanthema, is a self-limiting disease process characterized by a ulcerative dermatitis affecting the skin of the vulva or penis.[141] Equine herpesvirus-2 (EHV-2), a beta-herpesvirus, is known as equine cytomegalovirus because of its slow growth pattern and the persistence of viral shedding subsequent to infection.[142] The documentation of a specific pathologic process directly attributable to EHV-2 is lacking. However their is a high index of suspicion that an immunosuppressive condition exists, similar to the immune suppression associated with human cytomegalovirus infection.[143,144,145,146,147] A fifth, dissimilar equine herpesvirus, is reported to have been identified following a review of 51 field isolates of slowly replicating equine herpesviruses, by Browning and Studdert.[148] This viral isolate contained a significantly smaller genome than other EHV-2 isolates, although many of its' other characteristics were similar. The uniqueness of this virus remains to be corroborated.

In order to focus on the differentiation between EHV-1 and EHV-4, an understanding of the historical perspective of equine herpesvirus infection is necessary. In 1932, Dimock and Edwards isolated a herpesvirus from an aborted equine fetus from a mare in Kentucky.[149] Subsequently, the abortogenic nature of the virus was confirmed by inoculating pregnant mares with the virus and repeated isolation of the virus from fetal tissues, thus fulfilling Koch's

postulate.[150,151] A tremendous amount of research was directed toward the characterization of EHV-1, also known as "equine abortion virus". The respiratory and neurologic manifestations of the disease were subsequently elucidated and the development of various vaccines ensued. Several comprehensive reviews are available for a more in-depth review of the historical aspects of equine herpesvirus characterization.[152,153]

Kawakami et al. in 1962, were the first to present evidence suggesting that two distinct viral subtypes existed under the classification of EHV-1.[154] Strong epidemiologic evidence of the clinical and pathologic relevance of this postulate was recorded when the first confirmed outbreak of equine herpesvirus induced abortion occurred in Australia.[155] EHV-1 had been isolated from horses with respiratory disease for over 15 years, but EHV-1 was not recovered from an aborted equine fetus, in Australia, until 1977, despite a comprehensive surveillance program. Additional data continued to accumulate in support of this theory and in 1981, following the advent of restriction endonuclease technology, two distinct viruses classified as EHV-1 were identified. Restriction endonuclease analysis of the two field isolates was instrumental in the documentation of the genomic differences between these two viruses. The viruses initially known as EHV-1, subtypes 1 and 2, are now commonly referred to as EHV-1 and EHV-4, respectively.[156]

The correlation between EHV-4 and respiratory disease in the absence of

abortogenic and neurologic symptoms lead researchers to investigate the pathogenic characteristics of EHV-1 and EHV-4. EHV-4 replicates in the epithelium of the respiratory tract and is carried by macrophages to regional lymph nodes, however viremia does not develop. In contrast, EHV-1 infects and proliferates within the respiratory epithelium and endothelial cells. Circulating lymphocytes become infected as a result of viral replication in the endothelium and disseminate the infection throughout the body.[21]

2. Structure

Herpesviruses are double-stranded DNA viruses surrounded by a nucleocapsid with an amorphous zone known as the tegument located just underneath the proteinaceous envelope. Located on the surface of envelope are external projections composed of glycoproteins. The EHV-1 nucleocapsid is composed of six polypeptides with an additional eight polypeptides located in the tegument. For more detailed description of the individual architecture of equine herpesvirus-1 the reader is referred to the extensive discussion of this subject published by Allen and Bryans.[1]

3. Antigenic Characteristics

The principal antigenic components of EHV-1 identified are the envelope glycoproteins.[32,157,158] Immunogenicity, based on serologic reactivity, has not been associated with the polypeptides of the nucleocapsid and tegument.[159,160] The ability of viral gene products, synthesized in herpesviral

infected cells, to serve as targets for immune recognition has been illustrated in herpesviral infections of other species and similar events may occur as a component of the horses immune response to herpesviral infection.[161,162] Bridges and Ledger et al, identified 5 or 6 different polypeptides produced by EHV-1 infected cells which precipitated upon exposure to hyperimmune serum and therefore may be involved in the horses' immune response to EHV-1 infection.[163] Turtinen and Allen (1982) identified 11 or 12 distinct glycoproteins on the surface of the viral envelope of EHV-1 with six of these glycoproteins classified as major glycoproteins.[164] Variability in the number of glycoproteins isolated depends on the species origin of the cell line in which the virus is replicated and the method of analysis. Six glycoproteins, gp2, gp10, gp13, gp14, gp17/18, and gp21/22a are considered to have a role in the hosts immune response.[21] A number of studies have attempted to delineate the association of these glycoproteins with the seven major glycoproteins, gB, gC, gD, gE, gG, gH, and gI, described for herpes simplex virus-1 (HSV-1).[165,166,167,168,169] The value of this analogy is based on the large number of studies analyzing the complex interaction which occurs after entry of HSV-1 into a susceptible host. Three EHV-1 glycoproteins have been demonstrated be genetically similar to HSV-1 glycoproteins. Specifically, gp13 is the homologue of gC, gp14 is the homologue of gB, and gp 17/18 is the homologue of gE.[170,171]

In addition to their antigenicity, each glycoprotein mediates a specific

function. Adherence and penetration into host cells is mediated through gB.[172] Glycoprotein gB induces a delayed hypersensitivity reaction in addition to inducing antibody production.[173] Other unique features of these envelope glycoproteins includes complement binding by gC; induction of potent neutralizing antibodies by gD; the attachment to the Fc receptor of IgG by gE; and the type specificity of gG (reviewed in reference[174]).

B. Pathogenesis

1. Epidemiology

At least three distinct type of epidemics, respiratory, neurologic, and abortogenic, have been described in association with EHV-1 infection. The distinction between the pathogenesis of viral infection with EHV-1 and EHV-4 resulted in a reevaluation of viral specificity associated with previously documented disease outbreaks. Examination of viral isolates from 35 epidemics of equine respiratory disease in Kentucky for the years 1965 to 1985 revealed that 86% of the epidemics were attributable to EHV-4 infection.[21] For the 5 year period, 1985 to 1990, EHV-4 was identified in 100% of the respiratory epidemics in Kentucky in which an equine herpesvirus was isolated.[175] One abortogenic epidemic investigated in Kentucky since 1965, was associated with EHV-4 infection. The other 200 outbreaks were due to EHV-1.[21] Recently, EHV-4 was identified in the tissues of an aborted fetus.[176] EHV-1 was the primary pathogen isolated in all verified epidemics of equine herpesvirus induced neurologic signs

.[21] Therefore, the cumulative effects of EHV-1 infections appear to be associated with greater morbidity and mortality rates, and a lower prevalence rate than EHV-4 infections.

2. Clinical Syndromes

Viral respiratory disease is a common cause of illness in horses which infrequently results from EHV-1 infection. A distinction in the severity of clinical signs associated with EHV-1 and EHV-4 induced respiratory disorders has been identified and substantiated experimently. EHV-1 causes a high biphasic temperature elevation, leukopenia, nasopharyngitis, and tracheobronchitis. Clinical signs include depression, inappetence, and a serous nasal discharge.[177] Coughing, nasal mucosal congestion, and lymphadenopathy of the intermandibular and pharyngeal lymph nodes may also be observed. Secondary bacterial infections are a common sequela to EHV-1 infection, especially if severe pulmonary involvement develops.[178] EHV-4 generally results in a mild self-limiting infection of the upper respiratory tract with low grade temperature elevation.[179] The most important distinction between the clinical syndromes produced by EHV-1 and EHV-4 infection is ability of EHV-1 to become systemic and progress to abortogenic and neurologic disease.

An epidemic of contagious neurologic disease named "Infectious Paralysis of Horses" was first described by Comeny in 1888. [180] However, the definitive association between EHV-1 and the neurologic manifestations of the disease was

not made until 1966.[181] Myeloencephalitis results from endothelial cell infection, arterial thrombosis, and ischemic induced necrosis.[5,182,183] It is unknown if the vasculitis occurs as a consequence of an immune-mediated hypersensitivity reaction or as a result of thrombosis secondary to endothelial cell damage. Although horses of any age or sex can be affected with this syndrome, pregnant females have the highest incidence of EHV-1 induced neurologic disease.[6,184,185,186] Two theories have been proposed to explain the increased risk of neurologic disease associated with pregnancy. Pregnancy induced immunosuppression may predispose mares to a more widespread, severe infection. A predisposition to arterial thrombosis due to pregnancy induced hypercoaguability is another potential risk factor. Increases in fibrinogen, factor VIII:C, and von Willebrands factor activity occurs in mares for four months prepartum and five months postpartum.[187] This correlates with the period of greatest risk of paralysis.[188]

Considerable financial losses result from EHV-1 induced perinatal mortality which occurs as a consequence of abortions or the delivery of premature, critically ill neonates.[21] The average incubation period, from exposure to abortion, is 14 to 21 days, with a range of 9 to 120 days.[11,189] The pathogenesis of fetal infection requires development of maternal viremia.[190] Widespread endothelial cell infection results from viremia, spreading the infection to uterine and placental endothelial cells. Endothelial cell involvement progresses to the umbilical vessels

where fetal leukocytes become infected and transport the virus throughout the fetus.[191] Thrombosis of placental and fetal vessels eventually produces premature termination of the pregnancy and fetal death.

3. Transmission

Infection with EHV-1, in adult horses, is acquired by viral entry through the respiratory system. Inhaled viral particles attach to the nasal mucosa and replicates locally within epithelial and endothelial cells.[192] Direct exposure to contaminated fetal tissues can also result in infection, although the respiratory system is still the portal of entry. The distance of transport of EHV-1 particles through aerosol mediated transmission is small.[21] Therefore, stabling horses in crowded barns increases the incidence of the disease.

C. Diagnosis

Traditional diagnostic methods to detect EHV-1 infection consist of viral isolation in cell culture systems, a four-fold rise in virus-neutralizing antibody titer, and tissue staining with fluorescent antibodies.[21] Other serologic diagnostics, including complement-fixation, radial immunodiffusion enzyme assay (RIDEA), and enzyme-linked immunosorbent assay (ELISA), have been used to a limited extent.[193,194,195] The sensitivity of virus isolation and serologic diagnostic techniques is less than 80%.[21] Diagnosis of EHV-1 mediated abortions by serologic methods is not possible because the interval between maternal infection and abortion is often prolonged.[137] The recent development of two new, highly

sensitive diagnostic techniques, polymerase chain reaction and indirect immunoperoxidase staining may provide a more rapid and effective means of diagnosing EHV-1 infection.[196,197,198] This will assist in defining the prevalence and economic importance of the EHV-1 mediated diseases.

D. Latency and Recrudescence

Latency, the absence of viral replication or infectious particles in tissues which harbor the virus, is a distinguishing characteristic of members of the family Herpesviridae.[199] Clinical and experimental evidence exists supporting the role of latency in EHV-1 infections in horses. The ability of EHV-1 to establish latent infections which serve as a reservoirs for subsequent disease outbreaks was illustrated in an isolated herd of 197 Welsh mountain ponies. Evidence of EHV-1 infection was not detected in the herd until ponies were subjected to the stresses of weaning, castration, and relocation. After subjecting the ponies to these stress, EHV-1 was isolated from ponies of all ages.[200] Experimental, reactivation of latent infections in vivo, has been induced by parenteral corticosteroid administration.[201] Although EHV-4 was isolated once from neuronal tissue from a Thoroughbred mare with neurologic disease, evidence of neurotropism and latency within nervous tissue does not exist for EHV-1. Neurotropism is a common finding with other alpha herpesviruses such as herpes simplex and bovine herpesviruses. In vitro assays have demonstrated that EHV-1 could be isolated from PHA-treated buffy coat preparations, but not from unstimulated white blood

cells.[202] Subsequent work confirmed this finding and identified the cell population harboring the latent virus as T lymphocytes.[4] The mechanisms by which herpesvirus evade the immune system to establish latent infections is not completely understood. Evidence suggests that the cellular immune response may have a direct effect on the development of latency and recrudesce of herpesviral infections.[203]

E. Prevention and Control

Numerous vaccines have been developed in an attempt to control EHV-1 infections and to reduce the incidence of clinical signs. Since the advent of the inactivated EHV-1 Army-183[f] strain vaccine the incidence of equine herpesvirus induced abortions, in Kentucky, has declined from 9.8% to 4.5% of aborted fetuses.[21] The rate of fetal infection per year, detected in Kentucky, from vaccinated mares is 1.7%. Attenuated or killed virus vaccines lower the incidence of clinical signs, however the vaccines do not alter the rate of viral infection or incidence of viral shedding.[11] Viral infection and shedding can occur even in the presence of high circulating concentrations of virus-neutralizing antibodies. A greater reduction in the infection rate and incidence of viral shedding may occur after vaccination with modified live viruses.[204,205] Two different EHV-1 modified

[f] Pneumabort K, Fort Dodge Co., Fort Dodge, IA.

live-virus vaccines have been commercially available during the past 15 years.[g,h] However, the potential exists for the vaccine to become virulent and precipitate disease.[206] A recent study indicated that the incidence of viremia, viral shedding, and abortions, after challenge with a field strain of EHV-1, was not reduced by modified live-virus or inactivated EHV-1 vaccines.[207] A live virus vaccine with a thymidine kinase negative EHV-1 mutant stimulated the production of virus-neutralizing antibodies and reduced the incidence of respiratory disease, but did not alter the rate of viral shedding.[208] Prevention of the clinical symptoms of EHV-1 infection and eradication of infection will not be effective unless more effective forms of immunoprophylaxis are developed. Successful alteration of the immune response to reduce or eliminate EHV-1 infections can only occur if the equine immune response to EHV-1 is completely understood.

III. IMMUNE RESPONSE TO EQUINE HERPESVIRAL INFECTION

A. Introduction

The complexity of the multiple interactions comprising the immune response to viral infection dictates the necessity of repetitive experimentation,

---

[g] Rhinoimmune, Norden Laboratories, Lincoln, NE.

[h] Rhinoquin, Bio-Ceutic Laboratories, St. Joesph, MO.

both in vitro and in vivo, before a complete understanding of the host response is acquired. Immunologic responses to herpesviral diseases have been studied extensively in non-equine species, providing basic information regarding host reaction to herpesviral infection. Although in certain instances correlations may be deduced from experimentation in other species, this does not exclude the need for species-specific parallel studies. The purpose of this segment of the literature review is not only to recapitulate the sparse literature regarding the equine immune response to EHV-1, but to draw analogies with research conducted in other species relating to the immune response to herpesviral infection.

Immunity, the resistance to reinfection, is short lived for EHV-1 and immunologically experienced horses are subject to reinfection with an identical virus within 3 months after recovery.[209] Viremia accompanies reinfection which can result in abortion or neurologic disease. Attempts to correlate virus-neutralizing antibody titers with immunity to reinfection with EHV-1 have yielded unpredictable results.[138] Correlation of the host's immune status with virus-neutralizing antibody titers is complicated by the serologic cross-reactivity which occurs between EHV-1 and EHV-4.[210] Investigations on herpes simplex virus (HSV) indicate a wide range of host responses are involved in the immune response to infection, however the specific factors which constitute protective immunity have not been clearly defined.

B. Diagnostic Techniques

In vitro evaluation of the immune response does not provide an identical replication or analysis of events as they occur within the horse. Different in vitro assay systems examine distinctly different aspects of the immune response. The correlation between the in vitro analysis and in vivo response is not always evident. The accuracy of these analogies depends upon the nature of the pathogen and the ability of the assay system to mimic host conditions. In order to discern the relevance of previous research on the equine immune response to EHV-1 infection an understanding of the functional response analyzed by frequently used diagnostic techniques is necessary. Information regarding the specificity of the cell-mediated immune response, recognition of viral antigens, and the role of MHC-restriction in the context of in vitro assay systems has only recently been collected. As this information becomes available, it is often necessary to reevaluate conclusions reached in earlier work.

The vast majority of experimental studies on the equine immune response to EHV-1 infection analyze the production of EHV-1 binding antibodies.[211] Techniques evaluating serologic responses to EHV-1 infection include virus-neutralization, complement-fixation, radial immunodiffusion enzyme assay (RIDEA), and enzyme-linked immunosorbent assay (ELISA).[212,213] Although antibody production reflects exposure to EHV-1, antibodies alone do not provide protection against infection nor do they correlate with the horse's resistance to disease.[214] Experimental EHV-1 infection of pregnant mares with virus-

neutralizing antibodies did not result in any significant difference in the abortion rate.[215] Plasma cells, the end product of B cell differentiation and maturation, produce EHV-1 specific antibodies as a result of multiple interactions between lymphocytes and other antigen presenting cells. Intercellular communications, culminating in the production of immunoglobulins, are restricted by class II MHC molecules which requires processing of viral antigens through endosomal pathways. The biochemical characteristics of antigens processed in this fashion are distinctly different than exogenously processed antigens displayed in association with class I MHC molecules.[26,216]

Cellular immune responses to viral infections have been analyzed by three distinctly different methods; lymphocyte proliferation assay, cytotoxicity assays using autologous cells as targets, and cytotoxicity assays with heterologous target cells. Lymphocyte proliferation, in response to antigenic stimulation, is measured in vitro by the amount of tritiated thymidine incorporated into the DNA of replicating lymphocytes.[217] Equine lymphocytes have the capability to respond in vitro to mitogenic and antigenic stimulation, including viral antigens.[218,219] Activation of T cells results from the stimulation induced by recognition of a processed viral antigen to which the animal was previously sensitized. Upon activation, T cells produce cytokines, predominantly IL-2 and gamma-interferon, which enhance proliferation and cellular activity.[220,221,222,223] The interaction between viral antigens and T cells, required to induce lymphocyte proliferation,

is predominantly MHC class II restricted.[82] T cells designed to function in a class II restricted manner are CD4 -T helper cells whose primary activity is to enhance antibody production, although a small percentage of these cells may mediate cytotoxicity reactions.[81,82,83,84,85] Therefore, analysis of the cell-mediated immune response to viral infection through the use of lymphocyte proliferation assays reflects the hosts ability to respond to endosomally processed, class II restricted antigens.

Class I-restricted cytotoxic T lymphocytes (CD8) are generated as a result of cytokine activation in equine lymphocyte proliferation assays after day 5 or 6.[224] Incorporation of tritiated thymidine into lymphocytic DNA after day 5 of an in vitro proliferation assay may serve as an indirect reflection of the generation of class I restricted cytotoxic T lymphocytes. However this activation is cytokine dependent, non-specific activity.[225] Evaluation of the cell-mediated immune response to EHV-1 infection through the use of lymphocyte proliferation assays does not thoroughly analyze exogenously processed antigens expressed in combination with class I molecules since the standard assay period is 72 hours. The discrepancy in class restricted antigen processing stipulates that analysis of anti-viral CTL functions should be conducted with direct cytotoxicity assays which reflect class I and II restricted interactions as well as non-MHC restricted cytotoxicity. Non-MHC restricted cytotoxic responses are mediated by two effector cell populations, natural killer (NK) cells and a small percentage of CTL, many of

which bear the gamma-delta T cell receptor.[226,227,228] The in vivo significance of NK cells in immunity to naturally occurring herpesviral infections in humans has been highlighted by the several recent case reports of severe recurrent herpesviral infections in individuals lacking NK activity.[51,52,229]

Laboratory animal models of EHV-1 infection, using mice and hamsters, have been characterized. The development of laboratory animal models which mimic EHV-1 infection in the horse is important because of the difficulty in obtaining and maintaining horses without prior exposure to EHV-1, the expense of studying large numbers of horses, and the genetic variability between horses. Anderson and Goodpasture originally identified susceptibility to EHV-1 infection in Syrian hamsters.[230] Further evaluation of the characteristics of EHV-1 infection in Syrian hamsters were described by Doll et al.[231,232] Since the preliminary identification and characterization of the Syrian hamster as an animal model of EHV-1 infection, immune splenocytes, antiserum, and peritoneal macrophages have been identified as components of protective immunity in the hamster. Infection with EHV-1 in hamsters can be prevented by immunization with envelope glycoproteins, pretreatment with anti-EHV-1-glycoprotein monoclonal antibodies, and some antiviral drugs.[160,233,234] Intranasal infection in hamsters does not consistently reproduce infection, therefore in these models EHV-1 infection is induced by intraperitoneal injection. The liver is the primary site of virus replication after intraperitoneal injection and with sublethal injections, the

infection is resolved within 6 days. This is distinctly difference from the pathogenesis of the natural infection in horses.

Neonatal mice are susceptible to intracerebral injections of EHV-1, however there is little similarity to the naturally occurring disease.[235] A model of EHV-1 infection in an inbred strain of adult mice has recently been described which closely mimics the pathogenesis of the disease in horses.[236] Infection was established via intranasal inhalation of suspensions containing infective EHV-1 virions, resulting in viral replication in the respiratory epithelium and subsequent viremia. Abortion, premature parturition, and fetal death occur secondary to fetal infection when pregnant mice are infected with intranasal EHV-1.[237] This model of murine infection should prove valuable for future studies of the immune response to EHV-1.

C. Humoral Responses

Foals are dependent upon colostral transfer of EHV-1 neutralizing antibodies because the equine placenta is impervious to antibodies. Clinically normal foals ingesting adequate amounts of colostrum develop approximately the same concentration of EHV-1 specific neutralizing antibodies as the mare by 35 hours of age. These antibodies persist for an average circulation period of 180 days.[238] Horses less than 1 year of age do not develop high virus-neutralizing antibody titers after their first exposure to EHV-1, requiring repeated exposure to or infection with the virus before significant titers are obtained.[239,240]

Infection of adult horses with EHV-1 stimulates production of virus-neutralizing antibodies which are detectable within 8 days of primary infection and 5 days after reexposure to the virus.[241] Virus-neutralizing antibodies to EHV-1 persist for more than one year and it is uncommon to find adult horses which lack EHV-1 antibodies, because of the prevalence of the virus in the population.[9,11] The presence of virus-neutralizing antibodies does not confer resistance to infection, although the duration of viral shedding in nasal secretions and the severity of illness may be reduced when virus-neutralizing antibody levels are high.[211] Adult horses exposed to EHV-1 develop complement-fixing (CF) antibodies within 1 to 2 weeks after infection, with these antibodies persisting for a relatively short period of time, ranging from 2 to 5 months.[242,243] Complement-fixing antibodies do not respond anamnestically upon repeated viral infection and CF antibody titers have not been correlated with resistance to EHV-1 infection in most studies of experimental infection.[30,244] Edington and Bridges in 1990 observed that two experimental infections of ponies with EHV-1 5 months apart induced protective immunity to experimental EHV-4 infection 4 months later. The CF antibodies in this study were cross-protective between EHV-1 and EHV-4, while the virus-neutralizing antibodies were type specific, suggesting that CF antibodies may be associated with protective immunity.[245] The contribution of cell-mediated immunity, conferred by viral infection, to the resistance to reinfection was not evaluated in this study.

Because of the inadequate protection stimulated by whole virus vaccines and the immunogenicity associated with viral glycoproteins, a number of investigations have focused on the role of envelope glycoproteins in stimulation of the immune response to EHV-1 infection. The original study which demonstrated the antigenic capacity of the envelope glycoproteins was performed by Papp-Vid and Derbyshire in 1978. They immunized hamsters with either purified viral envelope or nucleocapsid. Preimmunization with the purified viral envelope resulted in a survival rate of 100% after an intraperitoneal injection of EHV-1. In a hamster model of a sublethal infection with EHV-1, Stokes et al., evaluated the protective capacity of anti-glycoprotein antibodies. The simultaneous administration of monoclonal antibodies specifically directed against the six different major glycoproteins (gp2, gp10, gp13, gp14, gp17/18, gp21/22a) resulted in complete protection against infection via intranasal viral challenge.[235] Administration of these six monoclonal antibodies to hamsters prior to intraperitoneal challenge resulted in 80% protection against infection. Analysis of the protection against infection, provided by individual antibodies, anti-gp13 (gC homologue) and anti-gp17/18 (gE homologue) provided 75% protection with intranasal challenge and 80% protection with intraperitoneal challenge. Anti-gp14 antibody (gB homologue) was 100% effective in preventing infection with an intra-peritoneal challenge of EHV-1 but only 20% effective against intranasal exposure to EHV-1. Shimimuzi et al., produced seven different monoclonal antibodies with

neutralizing activity to EHV-1.[246] In their hamster model of EHV-1 infection, a lethal dose of virus was introduced by subcutaneous or intraperitoneal injection, the monoclonal antibody corresponding to anti-gp13 was 100% effective in preventing death. The anti-gp14 monoclonal antibody was 75% effective in preventing death. The ability of gp13 and gp14 to stimulate protective immune responses in hamsters has recently been demonstrated.[235] Injection of hamsters with recombinant vaccinia virus expressing EHV-1 glycoproteins gp13 and gp14 resulted in protective immunity against a lethal intraperitoneal viral challenge.[247,248] These findings corroborate murine studies using HSV-1 which reflect gC is the principal antigen responsible for inducing protective immunity in lethal infective dose studies.[249,250]

The antigenicity of gp10, gp13, gp14, and gp17/18 has been demonstrated in EHV-1 challenged SPF foals. Antibodies to these four glycoproteins were rapidly detected following vaccination with inactivated EHV-1 in SPF foals, however they did not provide protection against infection upon subsequent viral challenge with EHV-1.[32] While this study demonstrated rapid antibody production to EHV-1 glycoproteins, antibody production did not provide protective immunity in horses.

In summary, EHV-1 viral glycoproteins stimulate virus-neutralizing and complement-fixing antibody production in horses and laboratory animals. Antibodies are protective against EHV-1 infection in hamsters, but not in

experimentally induced or naturally occurring infections in horses. Therefore, it appears that cellular immunity is an important component of resistance to EHV-1 infection.

D. Cellular Responses

1. Antibody-Dependent Cellular Cytotoxicity

Virus-specific antibodies are capable of binding to viral antigens on the surface of infected cells, allowing attachment of Fc receptor-bearing cells, resulting in direct cytolysis of the infected cell. Subpopulations of macrophages, PMN, and natural killer cells with receptors for the Fc portion of immunoglobulin molecules mediate this function.[113,251,252,253] The role of ADCC in vivo is in limiting the dissemination of virus infected cells prior to the release of newly synthesized virions, especially in the initial stages of viral replication when antibody concentrations are low.[122] The primary effector cell mediating this activity in horses is the neutrophil, although macrophages and natural killer cells also possess this function.[30,254]

The only experiment to assess the role of ADCC in EHV-1 infections was performed by Stokes and Wardley in 1988. In this study, two different in vitro assay systems were used to evaluate the ability of three different populations of equine peripheral blood leukocytes to mediate ADCC against EHV-1 infection in horses with a history of exposure to EHV-1. The three cell populations isolated were neutrophils, adherent mononuclear cells (macrophages), and non-adherent

mononuclear cells (lymphocytes and natural killer cells). The cytotoxic activity of the effector cells was determined with an 8 hour assay using ^{51}chromium labeled EHV-1 infected equine fetal kidney cells as the target cell. At an effector to target cell ratio of 100:1 the percent cytotoxicity was 90% for neutrophils, 45% for non-adherent mononuclear cells, and 30% for macrophages. However, the non-adherent mononuclear cell population mediated the most substantial ADCC in the in vitro plaque reduction assay. In this experiment equine fetal kidney cells infected with EHV-1 were overlaid with antiserum plus effector cells. The reduction in the number of viral plaques compared to a control inoculum was considered the measure of ADCC. The control plate yielded 111 viral plaques compared to 33 plaques for neutrophils, 6 plaques for macrophages, and 0 plaques for the non-adherent cell population. The horses from which these cells were obtained, were challenged with EHV-1 to ascertain the in vivo importance of ADCC. All animals succumbed to viral infection. This study indicates that ADCC against EHV-1 infection in horses is mediated by either neutrophils, macrophages, and natural killer cells and that ADCC does not adequately provide protective immunity against EHV-1 infection in horses.

2. Lymphocyte Cytotoxicity

Cytotoxic T lymphocytes are an essential component of the immune response to herpesviral infections. Diminished CTL function results in a prolonged recovery and an increased severity of disease.[255,256,257] The majority of CTL

recognize viral antigens in association with class I MHC molecules, consequently CTL interact with virus infected cells but not free virions.[258,259] Thus, the anti-viral functions of CTL are most important with viruses which spread by direct intercellular passage, rather than the extracellular route.[260] Depletion of CD8 cells results in death of the host with high-dose HSV challenge, however CD4 cells provided adequate protection in low-dose viral challenge.[256,261,262] Studies evaluating the significance of CTL in herpes simplex have shown that optimal protection from experimental infection in vivo requires the presence of both CD4 and CD8 cells.[263,264,265] Since the participation of CTL is a major component of the immune response to herpesviruses, it is important to identify the antigenic determinants recognized by CTL. The location of viral glycoproteins on the external surface of cell membranes makes them an obvious target. Indirect evidence indicates that HSV glycoprotein gG is recognized by CTL. Herpes simplex virus glycoproteins gB and gD which have been more extensively studied do not participate in CTL recognition. Proteins synthesized by HSV infected cells are capable of stimulating a protective CTL response which is class I restricted.[266] The significance of CTL recognizing viral gene products is highlighted by the correlation observed between individuals immune to herpesviral infection and the presence of CTL reactive with internally synthesized antigens.[267] In summary, CTL activity is important in protective immunity against herpes simplex infections. The presence of cellular immunity in horses

against EHV-1 infection has been demonstrated by the in vitro induction of lymphocyte proliferation induced with viral antigens. Thompson and Mumford analyzed EHV-1 antigen driven lymphocyte proliferation in foals with virus-neutralizing antibodies to EHV-1 and in SPF foals.[268] In both groups of foals, proliferative responses were displayed within 2 weeks of intranasal infection with EHV-1. Two peaks of activity occurred, at two to four weeks and eight weeks post-infection, for the SPF foals. Peak activity occurred at 3 weeks post-infection for foals with virus-neutralizing antibodies. In this study, lymphocyte proliferation in response to heat inactivated viral antigens was significantly greater for the SPF foals than foals with virus-neutralizing antibodies. However, the validity of the comparison between these two groups of foals must be questioned because of age differences between the groups. At the beginning of the study the SPF foals were 2 to 4 weeks of age, while the foals with circulating antibodies were 4 to 6 months of age. An age related reduction in lymphocyte activity of foals has been illustrated to occur between birth and 6 months of age by Magnuson, et al.[27] Thus, the lower lymphocyte proliferation values observed in foals with EHV-1 virus-neutralizing antibodies may be related to the increased age of foals comprising this group rather than the immune status of the foal.

Gerber et al., also evaluated EHV-1 antigen induced lymphocyte proliferation in foals and young horses.[269] In this study, 6 to 8 month old foals exhibited significant cell-mediated immunity to heat inactivated EHV-1 antigens,

as measured by the lymphocyte transformation test, after vaccination with a modified live equine herpesvirus 1 vaccine. Lymphocyte proliferation responses of 18 to 21 month old horses were low or absent in this study. Wilks and Coggins measured significant lymphocyte transformation responses to EHV-1 in three 12 to 24 month old SPF horses after experimental inoculation with EHV-1.[270] Thus, it appears that there is age related variability in the immune response to equine herpesviral infections.

Diminished CMI associated with pregnancy, culminating in EHV-1 abortion, has been identified by lymphocyte proliferation assays. Pachciarz and Bryans experimentally infected 21 mares, in the third trimester of pregnancy, with EHV-1 via intranasal inhalation.[271] Six of the 21 mares aborted 11 to 39 days after viral challenge. The 6 mares which aborted had significantly lower lymphocyte proliferation responses upon in vitro exposure to EHV-1 antigens, than the mares which maintained their pregnancy. Reduced CMI to EHV-1 was noted in five of six pregnant mares by Gerber et al. when they compared lymphocyte proliferation activity from pregnant mares to non-pregnant mares and stallions. However, evaluation of lymphocyte responses in pregnant mares with naturally occurring infections with EHV-1, by Dutta and Campbell, did not corroborate the findings of the studies by Gerber et al. and Pachciarz and Bryans.[272] In the study by Dutta and Campbell, no difference in lymphocyte proliferations responses was noted between pregnant mares which aborted and pregnant mares which delivered

healthy foals.

The lack of a direct relationship between antiviral cytotoxic responses and lymphocyte proliferation assays makes the in vivo importance of the findings of the studies performed with this technique difficult to assess. The duration of incubation of lymphocytes with EHV-1 antigens in these studies ranged from 3 to 6 days. Class I restricted CTL are not generated in lymphocyte proliferation assays until day 5 or 6. Equine herpesvirus-1 viral antigens used to activate lymphocytes in these assay systems are derived by heat inactivated or ultraviolet radiation of virions. Inactivated viral antigens do not undergo exogenous processing required prior to presentation with class I MHC molecules.[273,274] Therefore, the findings of these studies primarily reflect the proliferation of class II restricted CD4 cells - T helper cells. Since only an extremely small portion of class II restricted lymphocytes have cytotoxic activity, the results of these studies cannot be applied as a reflection of the horse's cytolytic capacity to EHV-1 infected cells.

Although cytotoxicity assays are the most accurate means of evaluating the function of CTL, a very small number of studies analyzing the direct cytotoxic activity against EHV-1 infected cells have been published. Stokes et al. measured the cytolytic activity of splenocytes, from EHV-1 infected hamsters, against autologous and xenogenic cells infected with EHV-1. They found that by day 6 post-infection, the spleens of infected animals contained virus-specific T

lymphocytes which could lyse either autologous or xenogenic target cells infected with EHV-1. Peak activity occurred on day 12 post-infection. In this study the adherent fibroblastic target cells were simultaneously infected with EHV-1 and labeled with ^{51}chromium (^{51}Cr) overnight, and then trypsinized to produce a cell suspension for the assay. The amount of spontaneous ^{51}Cr release was not mentioned in the article, however when the spontaneous release exceeds 25%, the results are transformed to have an erroneously high percent specific lysis.[275] Others have observed an excessively high percent spontaneous release with overnight ^{51}Cr labeling and the disruption of the cell membranes produced by trypsinization.[276,277] Therefore, it is possible that the high cytotoxic activity against EHV-1 infected cells reported in this study may be erroneously elevated due to high spontaneous release.

Wilks and Coggins in 1977 were the first to perform cytotoxicity assays against EHV infected target cells, with equine leukocytes.[278] In this study the strain of the virus used was not specified and since the distinction between EHV-1 and EHV-4 had not yet been delineated it is unknown if the virus was EHV-1 or EHV-4. They harvested peripheral blood mononuclear cells (PBMC) from 3 SPF horses after experimental infection with EHV and analyzed the cytotoxic activity against EHV infected equine fetal kidney cells in a 24 hour ^{125}iodine release assay. Cytotoxic activity of PBMC averaged 10 to 12% prior to infection and peaked at 40 and 60% on day 5 post-infection. Between days 5 and 10 post-

infection the cytotoxic activity declined to baseline levels with a second peak occurring around day 15. The cytotoxic activity of PBMC was noted in the presence or absence of autologous serum. However, freshly collected serum alone had an almost identical percent cytotoxicity as the PBMC. Other problems with the study included using the target cells in an adherent fashion which reduces their susceptibility to lysis, failure to remove the adherent mononuclear cell population (monocytes) which can either accentuate or inhibit the cytotoxic activity of lymphocytes, and the long length of the assay period producing high spontaneous release. Although, cytotoxic activity of equine PBMC against EHV infected target cells was evident in this study, the large number of confounding variables makes it difficult to determine the relevance of this study in light of the state of knowledge today.

The most comprehensive study to date on the cytotoxic activity of equine PBMC to EHV-1 was performed by Bridges and Edington in 1987.[67] In vitro cytotoxic activity of equine PBMC from five pony foals against EHV-1 and EHV-4 antigen labeled equine dermal fibroblasts was evaluated after in vivo experimental infection with EHV-4, in a 6 hour ^{51}Cr release assay. Cytotoxic activity was detected after incubating the PBMC for 65 hours in interleukin-2. Cytotoxic activity was determined at days 7 and 21 following the second infection with EHV-4. The cytolysis of EHV-4 labeled autologous fibroblasts was greater than for EHV-4 labeled allogenic fibroblasts or EHV-1 labeled autologous fibroblasts. The percent

cytotoxicity against the EHV-1 labeled autologous fibroblasts ranged from 0 to 20% at day 7 post-infection, returning to less than 10% by day 21 post-infection. The age of foals used in this study was not stated and as previously discussed there are age related variations the cytotoxic activity of foals. Although they were attempting to evaluate the activity of CTL, inactivated viral antigens were used to label the target cells. Inactivated viral antigens do not undergo exogenous processing which is required for presentation with class I MHC molecules.[280,281]

In conclusion, the role of cytotoxic T lymphocytes and other cells capable of mediating antiviral cytotoxicity in the immune response to equine herpesviral infections appears to be an important component of protective immunity. Additional studies are necessary to clarify these mechanisms in the horse and to evaluate the characteristics of the viral antigens which induce their activity.

IV. MONOCLONAL ANTIBODY IDENTIFICATION OF LEUKOCYTE CELL POPULATIONS

Monoclonal antibodies recognizing surface structures on lymphocytes and other blood cells can be used for phenotypic identification and numerical quantitation of different populations and subpopulations of cells. In addition to serving as antigenic receptors for monoclonal antibodies, surface structures on leukocytes are associated with specific functional properties of the cell population bearing that particular antigen. The characterization of effector cell populations

which mediate anti-viral activities is essential before a complete understanding of the intricacies of the immunologic response to viral infection is acquired. In recent years species-specific monoclonal antibodies have been developed for phenotypic analyses of equine leukocyte populations and cluster of differentiation (EqCD) numbers have been assigned to antibodies that define equine homologues of human and murine leukocyte markers.[74] Monoclonal antibodies of particular interest in the characterization of the immune response to equine herpesviral infections are those identifying T helper cells - EqCD4, cytotoxic T lymphocytes - EqCD8, pan T marker - EqCD5, and natural killer cells - EqT12.

Several different in vitro methods are available for evaluating the association between cytotoxic responses to EHV-1 infection and specific cell populations (Reviewed in reference [279]). Performing in vitro cytotoxicity assays using isolated peripheral blood cells and concurrent phenotypic analysis with monoclonal antibodies allows correlations to be drawn between the percent cytotoxicity and cell population numbers. Another method is negative selection of cells identified by complement-fixing monoclonal antibodies and their complement-mediated cytolysis prior to use in cytotoxic assays. The remaining cells are examined for a loss or enrichment in cytolytic function. Other means of negative selection include flow cytometric cell sorting and immuno-adsorption. A recently developed technique, immuno-magnetic separation, provides a mechanisms for positive selection of large numbers of purified cells.[280,281] In laboratory

animals, the effects of specific cell populations in vivo are evaluated by injecting monoclonal antibodies in a quantity sufficient to bind to all cells expressing compatible surface antigens. In vivo techniques utilizing monoclonal antibodies are not feasible for use in horses.

One experimental study has been performed analyzing the effect of EHV-1 infection on equine lymphocyte populations in specific pathogen free (SPF) foals.[282] Four SPF foals, 2 to 3 months of age, were infected with EHV-1 via intranasal inoculation. Primary infection resulted in a decline in the total lymphocyte counts, with EqCD4 and EqCD8 cell populations equally affected. Restitution of lymphocyte numbers during the recovery phase resulted in generation of a greater proportion of EqCD8 lymphocytes. Repeated challenge with EHV-1 2 months after the initial viral challenge did not result in reinfection, however EqCD8 numbers declined for several days immediately after infection. These findings implicate a potential role for EqCD8 lymphocytes in the immune response to EHV-1 infection. However, additional in vitro and in vivo studies are necessary before any conclusions can be drawn relating specific cell populations to immunity to infection.

MATERIALS AND METHODS

I. Animals

Ten mares of mixed breeds, ranging in age from 5 to 12 years, were used for this study. Horses were maintained at The Virginia Polytechnic Institute and State University (Blacksburg, Virginia), where they had been in residence for at least 1 year. All the horses were dewormed with ivermectin paste[i] and vaccinated for EHV-1 with a killed virus vaccine[j] four weeks prior to the experimental period. All mares used in this study had previously delivered live foals. One week prior to the study all horses underwent routine physical examinations, and hematologic and serum biochemical evaluation. Five mares had been verified as pregnant with a single conceptus, by transrectal ultrasound, at 18 to 25 days of gestation. The other five mares were non-pregnant. Reproductive status was confirmed at the beginning of the study by rectal palpation. All pregnant mares were in the seventh or eighth month of gestation during the experimental period. All mares remained healthy throughout the study.

II. Effector Cell Collection, Isolation, and Stimulation

Two hundred fifty ml of whole blood was collected by venipuncture into blood collection bags containing sodium citrate. The ratio of whole blood to sodium citrate was 9 to 1. The blood was maintained at room temperature until

---

[i] Eqvalan, Merck Co., Rahway, NJ.

[j] Pneumabort K, Fort Dodge Co. Fort Dodge, IA.

the peripheral blood mononuclear cells were isolated. Isolation was performed within three hours of collection. The horses were bled once weekly for 6 consecutive weeks. Blood sampled in weeks 1 and 2 was used for the preliminary characterization and development of the cytotoxicity assays. Blood sampled in week 3 was used for the experimental ^{51}chromium release cytotoxicity assays (one assay per horse). Labeling with monoclonal antibodies and flow cytometric characterization of lymphocyte subpopulations was performed with blood sampled in weeks 4 to 6 (three assays per horse).

The PBMC were isolated by density gradient centrifugation using Ficoll-Hypaque,[k] as previously described.[283] The whole blood was mixed with an equal volume of sterile 0.1 M phosphate buffered saline (PBS). Thirty-five ml of the whole blood-PBS mixture was layered over 15 ml of Ficoll-Hypaque in a sterile 50 ml plastic conical centrifuge tube (Corning). After centrifugation at 400 x g for 20 minutes, the mononuclear cells were collected from the plasma/Ficoll-Hypaque interface by aspiration through a small bore glass Pasteur pipet. The volume of PBMC-Ficoll-Hypaque suspension recovered was 10 ml or less. The PBMC were washed twice in 30 ml of 1 X modified Hanks balance salt solution (HBSS) without calcium or magnesium and centrifuged at 600 x g for 10 minutes. The supernatant was discarded and the PBMC were resuspended in RPMI-1640 cell culture medium with 5% fetal calf serum (FCS). Cell viability was determined

---

[k] Histopaque 1077, Sigma Chemical Co., St. Louis, MO.

by the trypan blue exclusion method. A cell population was considered viable if the percent of live cells was in excess of 90%. Morphologic characteristics of the cell population were examined after cytocentrifugation and Wright-Giemsa stain analysis. The final concentration of the cell suspension was 1.5 to 2.0 x 10^7 cells diluted into 3 ml of RPMI-1640 with 5% FCS. This suspension was placed in 10 cm^2 plastic petri dishes and incubated at 37^0 C in 5% CO_2 humidified air for 2 hours. Contaminating monocytes adhered to the plastic, as previously described,[284] allowing isolation of a non-adherent mononuclear cell population. Wright-Giemsa preparations of the non-adherent cell population (lymphocytes) were cytocentrifuged and evaluated with light microscopy.

One hundred μl of the lymphocytes were suspended in RPMI-1640 containing 10% FCS, was placed in each well of 96-well tissue culture treated, round-bottom microtiter plates (Corning). Each sample was run in triplicate for each concentration of cells, corresponding to 1 x 10^6 cells/well, 5 x 10^5 cells/well, and 2.5 x 10^5 cells/well. Human recombinant IL-2 (IL-2)[l] was diluted with RPMI-1640 containing 10% FCS to a concentration of 100 units of IL-2/ml. One hundred μl of IL-2 solution, equivalent to 10 units of IL-2, was added to each well of the cell suspension. The cells were incubated for 72 hours in a 5% $C0_2$ humidified atmosphere at 37^0 C. After incubation the plates were centrifuged at 500 x g for 10 minutes and the supernatant was discarded. The IL-2 stimulated cells were

---

[l] rIL-2, Genzyme, Cambridge, MA.

washed twice in 200μl of 0.1 M PBS and centrifuged at 55 x g for 10 minutes. The cells were resuspended in 100 μL of RPMI-1640 prior to assaying for cytotoxicity.

III. Preparation and Titration of EHV-1

Equine dermal fibroblasts[m] (EDF), an adherent cell line, were grown to 80% confluency in 150 cm^2 tissue culture flasks in Eagles Minimum Essential Media (EMEM) containing 15% FCS at 37^0 C in 5% CO_2 humidified air. The medium was removed and replaced with an inoculum representing 5.6 X 10^7 $TDIC_{50}$ of Kentucky D strain EHV-1[n] diluted to a total volume of 5 ml EMEM. The flasks were placed on a rocker plate at room temperature for 60 minutes. An additional 15 ml of EMEM containing 5% FCS was added to each flask and the flasks were incubated at 37^0 C in 5% CO_2 humidified air. The cytopathic effects were observed by phase contrast microscopy every 12 hours until over 75% of the cells demonstrated typical EHV-1 related cytopathic effects.[285] The incubation period was approximately 96 hours.

The replicated EHV-1 in tissue culture flasks were frozen at -70^0 C, allowed to slowly thaw to 25^0 C, and then refrozen. The freeze/thaw sequence was repeated three times, to lyse EDF, which resulted in the release of infective virus

---

[m] American Tissue Culture Collection, Rockville, MD.

[n] Dr. George Allen, Department of Veterinary, University of Kentucky, Lexington, KY.

particles. The suspension was placed into 50 ml conical centrifuge tubes and centrifuged at 500 x g for 20 minutes. The supernatant was removed and aliquots of 1 ml were stored at -70^0 C.

Each well of a flat bottom 96 well tissue culture treated microtiter plate (Fischer) was seeded with 5 x 10^4 EDF suspended in 100μl of EMEM containing 5% FCS and was then incubated for 24 hours. Seven concentrations of ten-fold serial dilutions of the stock virus ranging from 1 x 10^1 to 1 x 10^7 were prepared in EMEM. Each dilution of stock virus was inoculated in aliquots of 100μl onto 4 wells of EDF. The plates were incubated at 37^0 C in 5% CO_2 humidified air and observed for cytopathic effects, by phase contrast microscopy, every 12 hours for 96 hours. Titer of the virus was expressed in median tissue infective culture dose ($TCID_{50}$).[286]

IV. ^{51}Chromium Release Assay

The suspension of EDF were diluted to a concentration of 1 X 10^7 cells/5 ml of EMEM containing 5% FCS. Five ml suspensions of EDF were placed in 25 cm^2 tissue culture flasks (Corning) and incubated at 37^0 C in a 5% CO_2 humidified air for 1 hour, allowing the EDF to adhere to the tissue culture flasks. The supernatant was withdrawn and the cells were inoculated with multiplicities of infection (MOI) of 0.1, 1.0, and 10.0, respectively of EHV-1 suspended in 1 ml of EMEM. Multiplicity of infection denotes the number of infective virus particles (virions) per cell. The flasks were placed on a tilting plate at room temperature for

60 minutes to allow viral infection of the target cell population. The supernatant was discarded and replaced with 5 ml of EMEM containing 5% FCS. Viral infection of the EDF was allowed to proceed for 18 hours at 37^0 C in 5% CO_2 humidified air. The supernatant was aspirated from the flasks and discarded after incubation. The EHV-1 infected target cells were removed from the flask by adding 1.5 ml of 0.5% trypsin, waiting 3 minutes until the cells were loosened, and then dislodging the cells by washing the flask with 5 ml EMEM. The cells were washed once with 10 ml EMEM in a 15 ml conical centrifuge tube, followed by centrifugation at 300 x g. The cells were resuspended in 5 ml of EMEM with 150 μC of sterile sodium ^{51}Chromate°. Chromium labeling was permitted to proceed for one hour at 37^0 C in 5% CO_2 humidified air with agitation every 5 minutes to ensure adequate dispersion of the EDF. The chromium labeled EDF were washed twice in EMEM and adjusted to 5 x 10^4 cells/ml in EMEM. Non-infected EDF were labeled simultaneously using the same protocol. The chromium labeled, EHV-1-infected and non-infected EDF, were the target cell population.

^{51}Chromium labeled, EHV-1 infected or non-infected EDF, were added to the lymphocytes (effector cells) in 96 well plates described in section II. The effector to target cell ratios of 200:1, 100:1, and 50:1 were achieved by adding to 5 X 10^3 target cells to 1 X 10^6, 5 X 10^5, and 2.5 X 10^5 effector cells. Lymphocytes, before and after IL-2 stimulation, were evaluated for cytotoxicity against EHV-1

° New England Nuclear, Wilmington, DE.

infected EDF and non-infected EDF. Each of these analyses was performed in triplicate. The maximal release of ^{51}Chromium was determined by adding 100μl of 1.0% Triton X-100 to 5 X 10^3 target cells. The amount of spontaneous chromium release was assessed by adding 100μl of chromium labeled target cells (5 X 10^3) to 100μl of EMEM. Controls using chromium labeled and non-labeled EDF, for both the infected and non-infected EDF were concurrently analysed. The assay was allowed to proceed for 4 hours at 37^0 C in 5% CO_2 humidified air. The 96-well plates were centrifuged at 500 x g for 8 minutes. A supernatant collection system[p] was used to collect the supernatant containing the released chromium which allowed retention of unlysed chromium labeled target cells in the bottom of the well. The amount of radioactivity, expressed as counts per minute (cpm) was determined with a Beckman 5500 gamma counter. The percent cytolytic activity was computed from the percentage of chromium released into the supernatant. The formula employed to derive the percent release for each sample set was:[287]

$$\frac{\text{cpm release/sample - cpm spontaneous release}}{\text{cpm maximal release - cpm spontaneous release}} \times 100\%$$

V. Serum Virus-Neutralizing Antibodies

Serum samples were obtained from all 10 mares 10 weeks after

[p] Supernatant Collection System, Skatron, Sterling, VA.

vaccination which corresponded to the end of the study. Virus-neutralizing antibody titers for EHV-1 were determined at New York State Diagnostic Laboratory, Cornell University using previously described procedures.[288]

VI. Surface Marker Analysis

Lymphocytes were obtained from all 10 mares on three different occasions. Isolated cells were suspended in HBSS and adjusted to a concentration of 1 x 10^7 cells/ml and 100μl aliquots were placed into 6 individual wells of 96 well microtiter plates (1 X 10^6 cells/well). Five mouse-anti-equine monoclonal antibodies [EqT12 (natural killer cell-like),[q] and HT14A (EqCD8),[q] HB61A (EqCD4),[q] HT23A (EqCD5),[q] and HB88A[r] (EqCD3)] were diluted to concentrations of 10μg/ml in HBSS containing 1% gamma globulin-free horse serum and 0.1% sodium azide.[74] One hundred μl of the diluted antibody was added to 1 X 10^6 cells and incubated at 4^0 C for 30 minutes. The cells were centrifuged at 500 x g for 10 minutes, supernatant discarded, and washed twice with HBSS. The cell pellet was collected after centrifugation and reconstituted with 100μl HBSS. Goat anti-mouse fluorescein conjugate[s] was diluted 300:1 into HBSS containing 1% gamma globulin-free horse serum and 0.1% sodium azide. One hundred μl of the diluted conjugate was added to each well. The plates were incubated at 4^0 C for 30

---

[q] Veterinary Medical Research and Development, Pullman, WA.

[r] Dr. W. C. Davis, Washington State University, Pullman, WA.

[s] Accurate Chemical Co., Westbury, NY.

minutes. After incubation the plates were centrifuged at 500 x g for 10 minutes, supernatant discarded, and the cells were washed twice in HBSS. The cells were fixed in 200 μl of 3% paraformaldehyde for 30 minutes at 4^0C. After incubation the plates were centrifuged at 500 x g for 10 minutes and the supernatant discarded. The cells were washed twice in HBSS and resuspended in 200μl of HBSS and stored in 96 well plates at 4^0 C until evaluation by flow cytometry. All flow cytometric evaluations were performed within 48 hours of fixation.

Controls were simultaneously prepared for each horse by incubating 1 X 10^6 lymphocytes diluted in 100 μl HBSS with 100μl of goat anti-mouse fluorescein conjugate. After incubation at 4^0 C for 30 minutes the cells were washed twice in HBSS and fixed as the labeled cells described above.

All samples were analyzed with an EPICS V, model 752, flow cytometer[t] equipped with a 5 watt, INNOVA 90 laser[u]. Laser emission at 488 nm was used to excite the immunofluorescent dye and to produce light scattering. Fluorescence was measured using a 525/10 nm bandpass filter and collected as the logarithmic interval. A minimum of 10,000 cells were counted for each sample and the resulting histograms were analyzed on a MDADS computer[k]. Cells displaying fluorescent intensities above the upper limit of the negative control distribution were considered positive. Fluorescence intensity was expressed as the mean

---

[t] Coulter Electronics Co., Hileah, FL.

[u] Coherent, Palo Alto, CA.

channel number for the fluorescence peak in the histogram.

VII. Statistical Analysis

The effects of EHV-1 infection, IL-2 stimulation, pregnancy, and effector to target cell ratio on the percent lymphocyte cytotoxicity were determined, using the General Linear Models procedure of the SAS System (SAS Institute, Inc. Cary, NC).[289] The arcsine square root transformation was performed on the data before each analysis to stabilize the variances. The first analysis was performed on the percent cytotoxic activity of lymphocytes obtained from non-pregnant mares. Factors of interest were: EHV-1 infection (+/-), IL-2 stimulation (+/-), and effector to target cell ratio (200:1, 100:1, 50:1). The second analysis was performed on EHV-1 infected EDF. Factors of interest were: pregnancy status (pregnant or non-pregnant), IL-2 stimulation (+/-), and effector to target cell ratio (200:1, 100:1, 50:1). Consequently, each analysis was performed as a three way analysis of variance.

The Pearson product moment correlation coefficient was calculated to determine the degree of association between percent cytotoxicity and either virus-neutralizing antibody titers or percentage of phenotypically identified lymphocytes. A Student's paired t-test was used to compare the mean differences in percentage of phenotypically identified lymphocytes between the freshly isolated and IL-2 stimulated lymphocytes. For all tests, significance was considered at the level of $p \leq 0.05$.

RESULTS

Isolation of Effector Cell Population

A non-adherent population of PBMC was isolated from 250 ml of whole blood. The number of cells retrieved ranged from 4 X 10^7 to 10 X 10^7 cells per collection. A typical cell population was 95 to 98 percent viable based on trypan blue exclusion method. Morphologic evaluation of cytocentrifuged, Wright stained preparation of the isolated cell population by light microscopy consistently revealed greater than 95% of the cells were lymphoid cells. After a 72 hour incubation with IL-2, cell viability was estimated to be 60 to 80% as determined by trypan blue exclusion method.

Replication of EHV-1

The titer of the EHV-1 stock virus was 5.6 X 10^7 $TCID_{50}$/ml. One ml of the stock virus was inoculated into 150 cm^2 flasks, containing EDF grown to 75 to 80% confluency, to produce the virus used in the cytotoxicity assays. Titers of virus lots produced ranged from 1 X 10^4 to 6 X 10^6 $TCID_{50}$/ml. Preparations containing 1 X 10^5 $TCID_{50}$/ml or greater were used.

Chromium Release Assay

Preliminary experiments were conducted to ascertain the appropriate $TCID_{50}$ of EHV-1 to incubate with 1 X 10^7 cells to result in adequate antigen expression necessary for effector cell recognition, in a high proportion of infected cells without cytopathic effects. This was important to maintain the amount of

spontaneous release between 5% and 25%. When the spontaneous release exceeds 25%, the results are transformed to have an erroneously high percent cytotoxicity.[275] The spontaneous release in the assays was consistently less than 20% (data not shown). The assay was performed with freshly isolated lymphocytes from non-pregnant mares. The infection was allowed to proceed until the initial appearance of CPE, which occurred 18 hours after viral infection. Previous studies using HSV-1, a virus with growth characteristics similar to EHV-1, have demonstrated optimal cytotoxicity results when the cells are infected with an MOI of 1 to 3 and ^{51}Cr labeling occurs after trypsinization for 60 to 120 minutes.[290,291,292] Maximum cytotoxicity resulted after an 18 hour infection when EDF were infected with EHV-1 at an MOI equal to 1 (figure 1). Therefore, in all subsequent experiments an MOI of 1 was used.

Specificity of the cytotoxic response was established by comparing the results of freshly isolated and IL-2 stimulated lymphocytes from 5 non-pregnant mares against non-infected EDF and EHV-EDF (Table 1). The percent cytolysis of the EHV-EDF was significantly ($p \leq 0.0001$) greater than the non-infected EDF by both the freshly isolated and IL-2 stimulated equine lymphocytes. Variation of the effector to target cell ratios did not result in a significant ($p=0.0504$) alteration in the percent cytolysis of EHV-EDF or EDF.

As measured by the ^{51}chromium release assay using EHV-EDF, significant ($p \leq 0.0001$) differences were found between the cytolytic activity of lymphocytes

from pregnant mares and non-pregnant mares (Table 2). This difference in percent cytotoxicity within the groups (pregnant mares and non-pregnant mares) was significantly increased by IL-2 stimulation of the lymphocytes ($p \leq 0.0001$), however there was no significant ($p=0.0591$) effect of altering the effector to target cell ratio.

Virus-neutralizing antibody titers were analyzed to determine if a correlation existed between either freshly isolated lymphocytes or IL-2 stimulated lymphocytes from pregnant and non-pregnant mares. The titers of EHV-1 virus-neutralizing antibodies ranged from 1:48 to 1:192. There was no correlation between virus-neutralizing antibody titers and percent cytotoxicity for freshly isolated cells ($p=0.19$) or IL-2 stimulated cells ($p=0.42$).

Phenotypic characterization of the isolated cell population was performed as an adjunct to the functional analyses (Table 3). Freshly isolated lymphocytes from pregnant mares had a greater percentage of cells identified as EqT12+ than freshly isolated lymphocytes from non-pregnant mares. After 72 hours of IL-2 stimulation there were no differences in the percentage of cells identified as NK-like cells (EqT12), EqCD3 T lymphocytes (HB88A), EqCD4 T helper cells (HB61A), EqCD8 CTL (HT14A), or EqCD5 T lymphocytes (HT23A) between the pregnant and non-pregnant mares. The 72 hour incubation with IL-2, for pregnant mare lymphocytes, resulted in a significant ($p \leq 0.03$) increase in the percentage of cells identified as EqCD3 T lymphocytes (HB88A). The percentage of cells from

pregnant mares identified as NK-like (EqT12) declined ($p \leq 0.01$) during the incubation with IL-2. Additionally, there was a smaller percentage ($p \leq 0.03$) of freshly isolated lymphocytes from non-pregnant mares identified as NK cell-like (EqT12) when compared to freshly isolated lymphocytes of pregnant mares.

In an effort to define the cell population mediating the cytolytic effect of equine lymphocytes, the correlation between percent cytotoxicity and the percentage of cells identified by different surface markers was analysed. The percentage of freshly isolated cells from non-pregnant mares identified as EqCD3 T lymphocytes (HB88A) negatively correlated with the percent cytotoxicity ($r = -0.70$). The percent cytotoxicity negatively correlated with percentage of IL-2 stimulated cells from pregnant mares identified as NK-like (EqT12) ($r = -0.70$), EqCD5 lymphocytes (HT23A) ($r = -0.56$), and EqCD3 lymphocytes (HB88A) ($r = -0.62$).

DISCUSSION

In this study, we characterized an in vitro ^{51}Cr release assay using a suspension of equine dermal fibroblasts infected with EHV-1 and demonstrated EHV-1 associated cellular cytotoxicity from lymphocytes isolated from adult horses. The primary problem encountered during the development of the assay was excessive spontaneous ^{51}Cr release resulting from the rapid cytolytic activity of the virus and from the trypsinization necessary for suspension of the EDF. Spontaneous ^{51}Cr release in excess of 25% results in an erroneously high percent specific lysis.[275,293] In order to minimize the spontaneous ^{51}Cr release, previous studies have labeled target cells with heat-inactivated viral antigens. However, inactivated viral antigens are processed through different intracellular pathways than live viruses or recombinant vectored virus antigens.[280,281,294] This may lead to in vitro evaluation of cytotoxic activity which is not relevant in vivo where direct endothelial cell infection produces expression of endogenously synthesized viral antigens.[295] Initial attempts to develop an assay were based on the protocol described by Stokes, et al. in which hamster fibroblasts were simultaneously infected with EHV-1 and labeled with ^{51}Cr during an overnight incubation.[105] Equine dermal fibroblasts prepared following this regimen yielded a percent spontaneous ^{51}Cr of 60 to 70%. Additional efforts to minimize the percent spontaneous release revealed that shorter periods of ^{51}Cr labeling after viral infection reduced the spontaneous ^{51}Cr release to an acceptable level of less than

25%.

Cellular suspension is essential for maximal lymphocyte cytotoxic activity in 4-hour ^{51}Cr release assays.[44] Cytolysis of adherent target cells has slower reaction kinetics, requiring an 18 hour assay period and subsequent analysis of a different effector cell population (natural cytotoxic cells).[296] Preliminary investigations indicated that trypsinization prior to ^{51}Cr labeling resulted in a large amount of spontaneous release.[297,298] Chromium labeling of the target cells after viral infection and trypsinization impeded the uptake of ^{51}Cr into cells damaged by either viral replication or trypsinization. By diminishing the level of spontaneous ^{51}Cr release we were able to utilize a non-adherent target cell which had been directly infected with live EHV-1. The use of an adherent target cell (EDF) in a non-adherent manner permitted measurement of maximal lymphocyte cytolytic activity with rapid reaction kinetics. The notable features of our assay are EHV-1 infection of EDF with an MOI of 1 for 18 hours, trypsinization, then ^{51}Cr labeling for 1 hour immediately prior to analysis, retaining the cells in suspension.

The cytotoxic activity of both the freshly isolated and IL-2 stimulated lymphocytes from non-pregnant mares was consistently greater against the EHV-1 infected fibroblasts than the non-infected fibroblasts (Table 1). The non-infected fibroblasts served as a control, identifying the amount of non-specific cytolytic activity mediated by the lymphocytes. Demonstration of a higher percent lysis of EHV-1 infected fibroblasts than non-infected or control fibroblasts confirms that

the enhanced cytolytic activity was directly related to virus infection of the fibroblasts. Thus, the percentage of EHV-EDF lysed by the equine lymphocytes reflects EHV-1 associated cellular cytotoxicity.

Spontaneous cytolytic activity of the freshly isolated equine lymphocytes against EHV-1 infected EDF averaged around 5% prior to IL-2 stimulation. Incubation with IL-2 in vitro for 72 hours significantly increased the EHV-1 associated cytotoxic activity of the lymphocytes. Interleukin-2 is a potent inducer of cytolytic activity through increased production of cytolysins and enhanced reaction kinetics.[299,300] The cell population generated by a 3 to 7 day in vitro incubation with IL-2 is known as lymphokine activated killer cells (LAK).[301] In other species LAK arise from diverse populations of lymphoid cells including natural killer cells and cytotoxic T lymphocytes.[302,303,304] We were unable to discern a single phenotypically distinct cell population mediating the EHV-1 associated LAK cytolytic activity, as there was no direct correlation between the percent cytolysis and the percentage of cells phenotypically identified. This inability to define a single antiviral LAK phenotype probably reflects the normal heterogeneity of equine LAK.

The diminished resistance to disseminated EHV-1 infection associated with advancing pregnancy in horses has remained unresolved despite investigations into the etiopathogenesis of this phenomena. A low level of spontaneous cytotoxic activity against EHV-1 infected EDF could consistently be demonstrated from

lymphocytes isolated from adult non-pregnant mares. This EHV-1 associated spontaneous cytotoxicity was significantly greater than that from lymphocytes of pregnant mares. The reduced lymphocyte cytotoxicity observed in pregnant mares may reflect a decreased immune response possibly associated with the increased susceptibility to EHV-1 infection during pregnancy in horses. This would correlate with the findings of previous studies on EHV-1 associated cell-mediated immunity using lymphocyte proliferation assays.[271,272] Elevated concentrations of estradiol and progesterone which occur during pregnancy, are known to suppress cell-mediated cytotoxicity in other species.[305] It is plausible that a similar hormonally-mediated suppression in cellular cytolytic activity occurs in mares during pregnancy which can be equated with the reduced EHV-1 associated cytotoxicity in our study.

Interestingly, the LAK activity against the EHV-1 infected EDF was greater for lymphocytes from pregnant mares than from non-pregnant mares. This contrast was not paralleled by any dissimilarity in lymphocyte phenotypes. Immunosuppressive factors have been identified in the sera of other species during pregnancy which block IL-2 production by T cells, but do not alter LAK activity upon exposure to IL-2.[306,307] Impaired production of IL-2 has been correlated with recurrent herpes simplex virus infection and bovine herpes virus.[308,309] Therefore, it is possible that the lower cytolytic responses to EHV-1 from the pregnant mare lymphocytes upon isolation may result from impaired

production of IL-2 rather than an alteration in lymphocyte phenotypes or suppressor cell activity. The possibility of reduced IL-2 production during pregnancy in horses is an interesting concept which warrants further analysis as it may provide a clinical indication for the use of immunostimulatory pharmaceuticals in pregnant mares exposed to EHV-1.

Investigations into alterations in the percentages of lymphocyte subsets during pregnancy in other species indicates that most lymphocyte populations remain constant throughout pregnancy.[310,311] The findings of our study are in agreement with this, indicating that any changes in lymphocyte activity during pregnancy in horses are not a consequence of changes in lymphocyte phenotypes. The only difference in the percentage of phenotypically identified freshly isolated cells was a slight increase in the percent of natural killer cells in pregnant mares. The percentage of freshly isolated lymphocytes immunophenotypically identified was similar to that determined for horses in previous studies.[29,312,313] For both groups of horses the percentage of lymphocytes reacting with monoclonal antibodies identifying common T cell antigens (EqCD5, EqCD3) and T helper cells (EqCD4) increased during the genesis of LAK activity, while the percentage of CTL and natural killer-like cells remained relatively constant. This is consistent with the well characterized T cell mitogenic properties of IL-2.

Virus-neutralizing antibody titers were determined from serum samples

obtained from all the mares 10 weeks after vaccination. Virus-neutralizing antibody titers did not correlate with the percent cytotoxic activity before or after IL-2 stimulation. The vaccine used was a killed virus EHV-1 vaccine which is known to stimulate antibody responses, but it's efficacy in stimulating cytotoxicity has not been evaluated. The lack of correlation between virus-neutralizing antibody concentrations and cytotoxicity demonstrates that measurement of serum antibodies cannot be used as a substitute for evaluation of EHV-1 associated cytotoxic activity. Similar virus-neutralizing antibody titers were present between the pregnant and the non-pregnant mares. Antibody responses in humans remain intact during gestation while cellular immune responses concurrently decrease. The findings of this study suggest that a similar situation occurs in horses. Thus improved immunoprophylaxis against EHV-1 infection during pregnancy should include development of vaccines which enhance cellular cytotoxic mechanisms.

CONCLUSIONS and SUMMARY

Cellular cytotoxicity from equine lymphocytes can be measured with a 4 hour chromium release assay using EDF infected with EHV-1 at an MOI of 1 for 18 hours, trypsinization, and ^{51}Cr labeling for 1 hour. This cytotoxic activity is enhanced by a 72 hour incubation with IL-2. The EHV-1 associated cytotoxicity was greater for freshly isolated lymphocytes isolated from non-pregnant mares than pregnant mares. The diminished cytolytic activity of the lymphocytes from pregnant mares was restored by IL-2 stimulation. A single phenotypically distinct cell population mediating either the spontaneous or the IL-2 induced cytotoxic activity could not be distinguished. Virus-neutralizing antibody titers do not correlate with cytotoxic activity.

LITERATURE CITED

1. Allen GP, Bryans JT. Molecular epizootiology, pathogenesis, and prophylaxis of equine herpesvirus-1 infections. Prog Vet Microbiol Immun 2:78-144;1986.

2. Bryans JT, Allen GP. Equine viral rhinopneumonitis. Res Vet Sci 5:837-847;1986.

3. Patel JR, Edington N, Mumford JA. Variation in cellular tropism between isolates of equine herpesvirus-1 in foals. Arch Virol 74:41-51;1982.

4. Scott JC, Dutta SK, Myrup AC. In vivo harboring of equine herpesvirus-1 in leukocyte populations and subpopulations and their quantitation from experimentally infected ponies. Am J Vet Res 44:1344-1348;1983.

5. Jackson T, Kendrick JW. Paralysis of horses associated with equine herpesvirus-1 infection. J Am Vet Med Assoc 158:1351-1358;1971.

6. Jackson TA, Osburn BI, Cordy DR, Kendrick JW. Equine herpesvirus-1 infection of horses: studies on the experimentally-induced neurologic disease. Am J Vet Res 38:709-719;1977.

7. Doll ER. Seasonal incidence and fetal age in equine abortion. Cornell Vet 42:505-509;1952.

8. Platt H, Singh H, Whitwell KE. Pathological observations on an outbreak of paralysis in broodmares. Equine Vet J 12:118-126;1980.

9. Doll ER, Bryans JT. Incubation periods for abortion in equine viral rhinopneumonitis. J Am Vet Med Assoc 141:351-354;1962.

10. Mumford JA, Rossdale PD, Jesset DM, et al. Serological and virological investigations of an equine herpesvirus-1 (EHV-1) abortion storm at a stud farm in 1985. J Repro Fert Suppl 35:509-518;1987.

11. Burrows R, Goodridge D, Denver MS. Trials of an inactivated equid herpesvirus 1 vaccine: challenge with a subtype 1 virus. Vet Rec 114:369-374;1984.

12. Miller-Edge M, Splitter G. Patterns of bovine T cell-mediated immune responses to bovine herpesvirus 1. Vet Immunol Immunopathol 13:301-

319;1986.

13. Martin S, Cantin E, Rouse BT. Cytotoxic T lymphocytes: Their relevance in herpesvirus infections. Ann NY Acad Sci 532:257-272;1988.

14. Finberg R, Hom R. The role of T cell immunity in infection with the herpes group virus. Year in Immunol 2:267-278;1986.

15. Rouse BT, Horohov DW. Cytotoxic T lymphocytes in herpesviral infections. Vet Immunol Immunopathol 6:35-66;1984.

16. Miller-Edge M, Splitter G. Detection of impaired T cell-mediated immune responses to herpesvirus (BHV-1) in cattle. Vet Immunol Immunopathol 13:1-18;1986.

17. Kuo YC, Lin CY, Cheng SF, et al. Impaired natural killer cytotoxicity during recrudescence of recurrent herpes simplex virus type 1 infection. Cancer Det and Prev 1:51-55;1987.

18. Fleisher G, Bolognese R. Persistent Epstein-Barr virus infection and pregnancy. J Infect Dis 147:982-986;1983.

19. Gill TJ, III. Immunity and pregnancy. CRC Rev Immunol 5:201-227;1985.

20. Gonik B, Loo LS, West S, Kohl S. Natural killer cell and antibody-dependent cellular cytotoxicity to herpes simplex virus-infected cells in human pregnancy. Am J Repro Immunol Microbiol 13:23-26;1987.

21. Loftin KC, Gonik B, Kuraman P. Natural killer cytotoxicity and antibody-dependent cell cytotoxicity to herpes simplex virus-infected target cells in murine pregnancy. Am J Repro Immunol Microbiol 17:53-56;1988.

22. Young E, Killam AP, Green JF. Disseminated herpes virus in pregnancy: Association with primary genital herpes in pregnancy. J Am Med Assoc 235:2731-2733;1976.

23. Bailey JE, Schacter BZ. Mechanisms of diminished natural killer cell activity in pregnant women and neonates. J Immunol 134:3042-3048;1985.

24. Dupont B, Hansen JA. Human mixed-lymphocyte culture reaction: Genetics, specificity and biological implications. Adv Immunol 23:107-202;1976.

25. Termijitelen A, van Leeuwen A, van Rood JJ. HLA-linked lymphocyte activating determinants. Immunol Rev 66:79-101;1982.

26. Brodsky FM, Guagliard LE. The cell biology of antigen processing and presentation. Ann Rev Immunol 9:704-744;1991.

27. Magnuson NS, Perryman LE, Wyatt CR, et al. Large granular lymphocytes from SCID horses develop potent cytotoxic activity after treatment with human recombinant interleukin 2. J Immunol 139:61-67;1987.

28. Hormanski CE, Truax R, Pouriau SS, et al. Equine lymphokine activated killer (LAK) cells lyse allogenic but not xenogenic tumor cells. FASEB J 5:A638;1991.

29. Bridges CG, Edington N. Genetic restriction of cytolysis during equid herpesvirus subtype 2 infection. Clin Exp Immunol 70,276-282;1987.

30. Stokes A, Wardley RC. ADCC and complement dependent lysis as immune mechanisms against EHV-1 infection in the horse. Res Vet Sci 44:295-302;1988.

31. Chong YC, Duffus WPH, Hannant D. Natural killer cells in normal horses and specific-pathogen-free foals infected with equine herpesvirus. Vet Immunol Immunopathol 33:103-113;1992.

32. Crabb BS, Studdert MJ. Comparative studies of the proteins of equine herpesviruses 4 and 1 and asinine herpesvirus 3: antibody response of the natural hosts. J Gen Virol 71:2033-2041; 1990.

33. Bienenstock J, Befus AD. Review: mucosal immunology. Immunology 41:249-270; 1980.

34. Clamp JR. The role of mucus secretions in the protection of the gastrointestinal mucosa. Curr Top Vet Med Anim Sci 12:389-397; 1981.

35. Clamp JR. Chemical aspects of mucus. Br Bull Med 34:25-27; 1978.

36. Neighbour PA, Bloom BR. Natural resistance to viral infections. Semin Infect Dis 3:272-293; 1980.

37. Leon MA, Norden A. Kinetics of equine complement. J Immunol 83:99-104; 1959.

38. Thomson GR, Mumford JA, Campbell J, et al. Serologic detection of equid herpesvirus-1 infections of the respiratory tract. Equine Vet J 8:58-65; 1976.

39. Cooper NR. The classical complement pathway: activation and regulation of the first complement component. Adv Immunol 37:151-216;1985.

40. Sissons JGP, Oldstone MBA, Schreiber RD. Antibody-independent activation of the alternative complement pathway by measles-virus infected cells. Proc Natl Acad Sci 77:559-562;1980.

41. Cooper NR, Nemerow GR. Complement, viruses and virus-infected cells. Springer Semin Immunopathol 6:327-347;1983.

42. Barta O, Barta V. The equine complement system. Proceedings of the Fourth International Conference on Infectious Diseases:189-199, 1978.

43. Ohmann HB, Babiuk LA. Induction of receptors for complement and immunoglobulins by herpesviruses of various species. Virus Res 9:335-342; 1988.

44. Reynolds CW, Ortaldo JR. Nature and Physiology of Natural Effector Cells. Immunophysiology: The Role of Cells and Cytokines in Immunity and Inflammation, Openheim JJ, Shevach EM, Oxford University Press, New York, NY:336-349; 1990.

45. Herberman RB, Holden HT. Natural cell-mediated immunity. Adv Cancer Res 27:305-377; 1978.

46. Welsh RM. Regulation of virus infection by natural killer cells. Nat Immun Cell Growth Regul 5:160-199; 1986.

47. Welsh RM. Natural killer cells and interferon. CRC Crit Rev Immunol 5:55-93; 1985.

48. Ennis FA, Beare AS, Riley D, et al. Interferon induction and increased natural killer cell activity in influenza infections in man. Lancet ii:891-893; 1981.

49. Colmenares C, Lopez C. Enhanced lysis of herpes simplex virus type 1-infected mouse cell lines by NC and NK effectors. J Immunol 136:3473-3480; 1986.

50. Habu S, Akamatsu K, Tamaoki N, Okumura K. in vivo significance of NK cells on resistance against virus (HSV-1) infections in mice. J Immunol 133:2743-2747; 1984.

51. Biron CA, Byron KS, Sullivan JL. Severe herpes virus infections in an adolescent without natural killer cells. N Eng J Med 320:1731-1735; 1989.

52. Ballas ZK, Turner JT, Turner DA, et al. A patient with simultaneous absence of "classical" natural killer cells ($CD3^-$, $CD16^+$, and $NKH1^+$) and expansion of $CD3^+$, $CD4^-$, $CD8^-$, $NKH1^+$ subset. J Allergy Clin Immunol 85:453-459; 1990.

53. Lopez C, Kirkpatrick D, Read SE, et al. Correlation between low natural killing of fibroblasts infected with herpes simplex virus type 1 and susceptibility to herpesvirus infections. J Inf Dis 147:1030-1035; 1983.

54. Wilks CR, Coggins L. In vitro cytotoxicity of serum and peripheral blood leukocytes for equine herpesvirus type 1 infected target cells. Am J Vet Res 38:117-121; 1977.

55. Saksela E, Timonen T, Ranki A, Hayry P. Morphological and functional characterization of isolated effector cell responsible for human natural killer activity to fetal fibroblasts and to cultured cell line targets. Immunol Rev 44:71-123;1979.

56. Edens LM. Unpublished results.

57. Timonen T, Saksela E. Isolation of human natural killer cells by discontinuous gradient centrifugation. J Immunol Methods 36:285-291; 1980.

58. Timonen T, Ortaldo JR, Herberman RB. Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. J Exp Med 153:569-582; 1981.

59. Reynolds CW, Timonen T, Herberman RB, et al. Natural killer (NK) activity in the rat. I. Isolation and characterization of the effector cells. J Immunol 127:282-287;1981.

60. Ortaldo JR, Herberman RB. Heterogeneity of natural killer cells. Ann Rev Immunol 2:359-394; 1984.

61. Herberman RB, Reynolds CW, Ortaldo JR. Mechanism of cytotoxicity by natural killer cells. Ann Rev Immunol 4:651-680; 1986.

62. Reynolds CW, Ortaldo JR. Natural killer activity: the definition of function rather than cell type. Immunol Today 8:172-174; 1987.

63. Ritx J, Campen TJ, Schmidt RE, et al. Analysis of T cell receptor gene rearrangement and expression in human natural killer clones. Science 228:1540-1543; 1985.

64. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogenic tumors. I. Distribution of reactivity and specificity. Int J Cancer 16:216-229; 1975.

65. Piontek GE, Taniguchi K, Ljunggren HG, et al. YAC-1 MHC class I variants reveal an association between decreased NK sensitivity and increased H-2 expression after interferon treatment or in vivo passage. J immunol 135:4281-4288; 1985.

66. Welsh RM. Natural killer cells and interferon. CRC Crit Rev Immunol 5:55-93; 1984.

67. Bridges CG, Edington N. Innate immunity during Equid herpesvirus 1 (EHV-1) infection. Clin Exp Immunol 65:172-181; 1986.

68. Yilma T, McGuire TC, Perryman LE. Preliminary characterization of equine interferons and their antiviral activities on bovine, ovine, and human cells. J Interferon Res 2:363-370; 1982.

69. Edington N, Bridges CG, Griffiths L. Equine Interferons following exposure to equid herpesvirus-1 or -4. J Interferon Res 9:389-392; 1989.

70. Edington N, Bridges CG, Griffiths L. Further investigations of equine interferons in vitro. J Interferon Res 9:385-387; 1989.

71. Edens LM. Unpublished results.

72. Benacerraf B. Role of major histocompatibility gene products in immune regulation. Science 212:1229-1238; 1981.

73. de Weck AL, Lazary S, Bullen S, et al. Determination of leukocyte histocompatibility antigens in horses by serologic techniques. Proc Fourth International Congress on Equine Infectious Diseases:221-227; 1978.

74. Kydd JH, Antczak DF. First international workshop on equine leukocyte antigens 12th-13th July, 1991: preliminary report. Equine Vet J Suppl 12:4-5; 1991.

75. Lazary S, Bullen S, Muller J. Equine leukocyte antigen system II. Serological and mixed lymphocyte reactivity studies in families. Transplantation 30;210215; 1980.

76. Bailey E. Segregation distortion within the equine MHC; analogy to a mouse T/t-complex trait. Immunogenetics 24:225-229; 1986.

77. Crepaldi T, Crump A, Newman M, et al. Equine T lymphocytes express class II antigens. J Immunogenetics 13:349-360; 1986.

78. Davis WC, Marusic S, Lewin HA, et al. The development and analysis of species specific and cross reactive monoclonal antibodies to leukocyte differentiation antigens and antigens of the major histocompatibility complex for use in the study of the immune system of cattle and other species. Vet Immunol Immunopathol 15:337-376; 1987.

79. Parnes JR. Molecular biology and function of CD4 and CD8. Adv Immunol 44:265-311; 1989.

80. Zinkernagel RM, Doherty PC. Restriction of in vitro T cell mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature (London) 248:701-701; 1979.

81. Moretta L, Mingari MC, Sekaly RP, et al. Surface markers of cloned human T cells with various cytolytic activities. J Exp Med 154:569-574;1981.

82. Meuer SC, Schlossman SF, Reinherz EL. Clonal analysis of human cytotoxic T lymphocytes: T4 and T8 effector cells recognize products of different major histocompatibility regions. Proc Natl Acad Sci USA 79:4395-4399;1982.

83. Meuer SC, Hussey RE, Hodgon JC, et al. Surface structures involved in target recognition by human cytotoxic T lymphocytes. Science 218:471-473;1982.

84. Spits H, Borst J, Terhorst C, de Vries JE. The role of T cell differentiation markers in antigen-specific and lectin-dependent cellular cytotoxicity mediated by T8+ and T4+ human cytotoxic T cell clones directed at class I and class II MHC antigens. J Immunol 129:1563- 1569;1982.

85. Kolaitis G, Doymaz M, Rouse BT. Demonstration of class II-restricted cytotoxic T lymphocytes in mice against herpes simplex virus. Immunology 71:101-106; 1990.

86. Tonegawa S. The molecules of the immune system. Sci Am 253:122-131; 1985.

87. Unanue ER. Antigen-presenting function of the macrophage. Ann Rev Immunol 2:395-428;1984.

88. Brown JH, Jardetzky T, Saper MA, et al. A hypothetical model of the foreign antigen binding site of class II histocompatibility molecules. Nature (London) 332:845-850;1988.

89. Smith KA. T cell growth factor. Immunol Rev 51:337-357;1980.

90. Sanders VM, Fernandez-Botran R, Coffman RL, et al. A single antigen specific B cell can conjugate either type 1 or type 2 helper T cell. Proc Natl Acad Sci USA 85:7724-7728;1988.

91. Cherwinski HM, Schumacher JH, Brown KD, et al. Two types of mouse helper T cell clones, III. Further differences in lymphoid synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific and monoclonal antibodies. J Exp Med 166:1229-1244;1987.

92. Coffman RL, Seymour BW, Lebman DA, et al. The role of helper T cell products in mouse B cell Differentiation and isotype regulation. Immunol Rev 102:5-28;1988.

93. Hirano T, Yasuwaka K, Harada H, et al. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature (London) 324:73-76;1986.

94. Mair TS, Batten EH, Stokes CR, Bourne FJ. The histologic features of the immune system of the equine respiratory tract. J Comp Pathol 97:575-586; 1987.

95. Mair TS, Stokes CR, Bournes FJ. Quantification of immunoglobulins in the respiratory tract secretions of the horse. Vet Immunol Immunopathol 14:187-203; 1987.

96. Montgomery PC, Khaleel SA, Kenney RM. Induction and characterization of equine secretory antibodies. Proceedings Fourth International

Conference on Equine Infectious Diseases:177-187; 1978.

97. Hannat D. Immune responses to common respiratory pathogens: problems and perspectives in equine immunology. Equine Vet J Suppl 12:10-18;1991.

98. Whitton JL, Oldstone MBA. Virus-induced immune response interactions: Principles of immunity and immunopathology. Virology, Second Edition; Fields BN, Knipe DM, Raven Press, New York, NY:369-381; 1990.

99. Doll ER, Bryans JT. A planned infection program for immunizing mares against viral rhinopneumonitis. Cornell Vet 53:249-262; 1963.

100. Holmes DF, Lamb LM, Anguish LM, et al. Live temperature-sensitive equine influenza A virus vaccine: efficacy in experimental ponies. Proceedings Fifth International Conference Equine Infectious Diseases, University of Kentucky Press, Lexington, KY:88-93; 1988.

101. Johnson PR, Feldman S, Thomson JM, et al. Comparison of long-term systemic and secretory antibody response in children give live attenuated, or inactivated influenza A vaccine. J Inf Dis 154:121-127; 1985.

102. Friedman HM, Cohen GH, Eisenberg RJ, et al. Glycoprotein C of herpes simplex virus 1 acts as a receptor for the C3b complement component on infected cells. Nature 309:633-635; 1984.

103. Smiley ML, Hoxie JA, Friedman HM. Herpes simplex virus type 1 infection of endothelial, epithelial and fibroblast cells induces a receptor for C3b. J Immunol 134:2673-2678; 1985.

104. Babiuk LA, Wardley RC, Rouse BT. Defense mechanisms against bovine herpesvirus. Relationship of virus-host cell events to susceptibility to antibody complement cell lysis. Infect Immun 12:1132-1137; 1975.

105. Stokes A, Allen GP, Pullen LA, Murray PK. A hamster model of equine herpesvirus type 1 (EHV-1) infection; Passive protection by monoclonal antibodies to EHV-1 glycoprotein 13, 14 and 17/18. J Gen Virol 70:1173-1183; 1989.

106. Belding ME, Klebanoff SJ. Peroxidase-mediated virucidal systems. Science 16:195-196; 1970.

107. Drutz DJ, Mills J. Immunity and Infection. Basic and Clinical Immunology, Sixth Edition, Stites DP, Stobo JD, Wells JV, Appleton and Lange, Norwalk, CT:167-185; 1987.

108. Mogensen SC. Role of macrophages in resistance to virus infections. Microbiol Rev 43:1-26; 1979.

109. Darlington RW. The role of equine macrophages in resistance to infection by equine herpesvirus 1. Proceedings Fourth International Conference on Infectious Diseases:129-139; 1978.

110. Halstead SB. Immune enhancement of viral infection. Prog Allergy 31:301-364; 1982.

111. Kohl S, Moore CM. Human antibody-dependent cellular cytotoxicity and natural killer cytotoxicity to herpes simplex virus-infected autologous and allogeneic cells. Immunology 48:187-193; 1983.

112. Kohl S. Antiviral cellular cytotoxicity of colostrum. Human milk in infant nutrition and health, Howell RR, Morris FM, Pickering LK, Charles C. Thomas Publishing:190-204; 1986.

113. Oleske JM, Ashman RB, Kohl S, et al. Human polymorphonuclear leukocytes as mediators of antibody-dependent cellular cytotoxicity to herpes simplex virus-infected cells. Clin Exp Immunol 27:446-453; 1977.

114. Kohl S, Starr SE, Oleske JM, et al. Human monocyte-macrophage-mediated antibody-dependent cellular cytotoxicity to herpes simplex virus-infected cells. J Immunol 118:729-735; 1977.

115. Shore SL, Melewicz FM, Gordon DS. The mononuclear cell in human blood which mediates antibody-dependent cellular cytotoxicity to virus-infected target cells. I. Identification of the population of effector cells. J Immunol 118:558-566; 1977.

116. Melewicz FM, Shore SL, Ades EW, Phillips DJ. The mononuclear cell in human blood which mediates antibody-dependent cellular cytotoxicity to virus-infected cells. II. Identification as a K cell. J Immunol 118:567-573; 1977.

117. Kohl S, Cahall DL, Walters DL, Schaffner VE. Murine antibody-dependent cellular cytotoxicity to herpes simplex virus-infected target cells. J Immunol 123:25-30; 1979.

118. Thorne KJI, Norman JM, Haydock SF, et al. Antibody-dependent cell-mediated cytotoxicity against IBR-infected bovine kidney cells by ruminant neutrophils: the role of lysosomal cationic protein. Immunol 53:275-282; 1984.

119. Rouse BT, Babiuk LA. Mechanisms of recovery from herpesvirus infections - a review. Can J Comp Med 42:414-427; 1978.

120. Shore SL, Cromeans TL, Romano TJ. Immune destruction of virus-infected cells early in the infectious cycle. Nature 262:695-696; 1976.

121. Shore SL, Cromeans TL, Norrild B. Early damage of herpes-infected cells by antibody dependent cellular cytotoxicity: relative roles of virus-specific cell-surface antigens and input virus. J Immunol 123:2239-2244; 1979.

122. Kohl S. Role of antibody-dependent cellular cytotoxicity in defense against herpes simplex virus infections. Rev Inf Dis 13:108-114; 1991.

123. Coignoul FL, Bertram TA, Cheville NF. Functional and ultrastructural changes in neutrophils from mares and foals experimentally inoculated with a respiratory strain of equine herpesvirus-1. Am J Vet Res 45:1972-1975; 1984.

124. Parnes JR. Molecule biology and function of CD4 and CD8. Adv Immunol 44;265-311; 1989.

125. Notkins AL. Immune mechanisms by which the spread of viral infections is stopped. Cell Immunol 11:478-483; 1974.

126. Rouse BT, Norely S, Martin S. Antiviral cytotoxic T lymphocyte induction and vaccination. Rev Infect Dis 10:16-33; 1988.

127. Zingernagel RM, Althage A. Antiviral protection by virus-immune cytotoxic T cells. Infected target cells are lysed before infectious virus progeny is assembled. J Exp Med 145: 644-651; 1977.

128. Cobbold SP, Jayasuriya A, Nash AA, et al. Therapy with monoclonal antibodies by elimination of T cell subsets in vivo. Nature 312:548-551; 1984.

129. Martin S, Rouse BT. The mechanisms of antiviral immunity induced by a vaccinia recombinant expressing herpes simplex virus type 1 glycoprotein D: clearance of local infection. J Immunol 138:3431-3437; 1987.

130. Gillis S, Gillis AE, Henney CS. Monoclonal antibody directed against interleukin-2: 1. Inhibition of T lymphocyte mitogenesis and the in vitro differentiation of alloreactive cytolytic T cells. J Exp Med 154:983-988; 1981.

131. Nabholz M, Erand F, Sekali R, et al. Regulation of IL-2 receptors expression by IL-2 and IL-1. Fed Proc 44:1304; 1985.

132. Capra JD, Kehoe JM. Hypervariable regions, idiotypy, and the antibody combining site. Adv Immunol 20:1-40; 1975.

133. Matis LA. The molecular basis of T-cell specificity. Ann Rev Immunol 8:65-82; 1990.

134. Mills KHG. Processing of viral antigens and presentation to class II restricted T cells. Immunol Today 7:260-263; 1986.

135. Townsend A, Bodmer H. Antigen recognition by class I-restricted T lymphocytes. Ann Rev Immunol 7:601-624; 1989.

136. Morrison LA, Lakacher AE, Brachiale VL, et al. Differences in antigen presentation to MHC class I- and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. J Exp Med 163:903-921; 1986.

137. Bryans JT, Allen GP. Herpesviral Diseases of the Horse. Herpesvirus Diseases of Cattle, Horses, and Pigs, Wittman G., Kluwer Academic Publishers, Boston, MA, 1989.

138. Roizman B. Herpesviridae: A Brief Introduction. Virology 2nd edition, edited by Fields B. N. and Knipe D. M. Raven Press, Ltd., New York, NY, 1990.

139. Roizman B, Carmichael LE, Deinhardt F, et al. Herpesviridae. Definition, provisional nomenclature, and taxonomy. Intervirology 16:201-217; 1981.

140. Studdert MJ. A brief review of studies of bovine and equine herpesviruses. Aust Vet J 66:401-402; 1989.

141. Bryans JT, Allen GP. In vitro and in vivo Studies of Equine 'Coital' Exanthema. Equine Infectious Diseases III, Bryans JT and Gerber H, S. Karger Publishing, New York,NY, 1973.

142. Harden TJ, Bagast TJ, Pascoe RR, Spradbrow PB. Studies on equine herpesvirus 5. Isolation and characterization of slowly cytopathic equine

herpesviruses in Queensland. Aust Vet J 50:483-488; 1974.

143. Browning GF, Studdert MJ. Epidemiology of Equine Herpesvirus 2 (Equine Cytomegalovirus). J Clin Microb 25:13-16;1987.

144. Dutta SK, Campbell DL. Pathogenicity of Equine Herpesvirus: In Vivo Persistence in Equine Tissue Macrophages of Herpesvirus Type 2 Detected in Monolayer Macrophage Cell Culture. Am J Vet Res 39:1422-1427; 1978.

145. Ames TR, O'Leary TP, Johnston GR. Isolation of Equine Herpesvirus Type 2 from Foals with Respiratory Disease. Comp Cont Education 8:664-670; 1986.

146. Wilks CR, Studdert MJ. Equine herpesviruses. 5. Epizootiology of slowly cytopathic viruses in foals. Aust Vet J 50:430-442; 1972.

147. McChesney MB, Oldstone MBA. Viruses Perturb Lymphocyte Functions: Selected Principles Characterizing Virus-Induced Immunosuppression. Ann Rev Immunol 5:279-304; 1987.

148. Browning GF, Studdert MJ. Genomic heterogeneity of equine beta herpesviruses. J Gen Virol 68:1441-1447; 1987.

149. Dimock WW, Edwards PR. Infections of fetuses and foals. Kentucky Agriculture Experiment Station, Bulletin 333, Lexington; 1932.

150. Dimock WW, Edwards PR. Is there a filterable virus of abortion in mares?. Kentucky Agriculture Experiment Statio, Bulletin 333, Lexington:297-301; 1933.

151. Dimmock WW, Edwards PR. The differential diagnosis of equine abortion with special reference to a hitherto undescribed form of epizootic in mares. Cornell Vet 26: 231-239; 1936.

152. O'Callaghan DJ, Allen GP, Randall CC. Structure and replication of the equine herpesviruses. Equine Infectious Diseases IV, Bryans JT and Gerber H, Veterinary Publications, Inc. Princeton, NJ; 1978.

153. Campbell TM, Studdert MJ. Equine herpesvirus type-1 (EHV-1). Vet Bull 53:135-146; 1983.

154. Kawakami Y, Kaji T, Ishizaki R, et al. Etiologic study on an outbreak of acute respiratory disease among colts due to equine rhinopneumonitis virus. Jap J Exp Med 32:211-229; 1962.

155. Studdert MJ, Blackney MH. Equine herpesviruses: on differentiation of respiratory from fetal strains of type 1. Aust Vet J 55:488-492; 1979.

156. Fitzpatrick DR, Studdert MJ. Immunologic Relationships Between Equine Herpesvirus Type 1 (Equine Abortion Virus) and Type 4 (Equine Rhinopneumonitis Virus). Am J Vet Res 45:1947-1952; 1984.

157. Bridges CG, Ledger N, Edington N. The characterization of equine herpes virus-1 infected cell polypeptides recognized by equine lymphocytes. Virology 63:193-198; 1988.

158. Turtinen LW, Allen GP. Identification of the Envelope Surface Glycoproteins of Equine Herpesvirus Type 1. J Gen Virology 63:481-485; 1982.

159. Papp-Vid G, Derbyshire JB. The protective antigens of equine herpesvirus type 1. Can J Comp Med 42:219-226; 1978.

160. Papp-Vid G, Derbyshire JB. The virus neutralizing activity of antibodies specific to the envelope and nucleocapsid of equine herpesvirus type 1. Can J Comp Med 43:231-233; 1979.

161. Reddehase MJ, Keill GM, Koszinowski VH. The cytolytic T lymphocyte response to the murine cytomegalovirus. II. Detection Of virus replication stage-specific antigens by separate populations of in vivo active cytolytic T lymphocyte precursors. Eur J Immunol 14:56-61; 1984.

162. Martin S, Cantin E, Rouse BT. Cytotoxic T Lymphocytes: Their Relevance in Herpesvirus Infections. Ann NY Acad Sci:257-272; 1988.

163. Bridges CG, Ledger N, Edington N. The characterization of equine herpes virus-1-infected cell polypeptides recognized by equine lymphocytes. Immunology 63:193-198; 1988.

164. Shimizu M, Satou K, Nishioka N. Monoclonal antibodies with neutralizing activity to equine herpesvirus-1. Arch Virol 104:169-174; 1989.

165. Killington RA, Yeo J, Honess RW, et al. Comparative analyses of the Proteins and Antigens Of Five Herpesviruses. J Gen Virology 37;297-310;

1977.

166. Vestergard BF. Herpes simplex virus antigens and antibodies. A survey of studies based on quantitative immunoelectrophoresis. Rev Infect Dis 2:899-913; 1980.

167. Kumel G, Kaerner MC, Levine M, et al. Passive immune protection by herpes simplex virus specific monoclonal antibodies and monoclonal antibody resistant mutants altered in pathogenicity. J Virology 56:930-937; 1985.

168. Balachandran N, Bacchetti S, Rawles WE. Protection against lethal challenge of BALB/c mice by passive transfer of monoclonal antibodies to five glycoproteins of herpes simplex virus type 2. Infect Immun 37:1132-1137; 1982

169. Dix RD, Pereira L, Baringer JR. Use of monoclonal antibody directed against herpes simplex virus glycoproteins to protect mice against acute virus-induced neurologic disease. Infect Immun 34:192-199; 1981.

170. Allen GP, Yeargan MR. Use of lambda gt11 and Monoclonal Antibodies To Map the Genes for Six Major Glycoproteins of Equine Herpesvirus 1. J of Virology 61;2454-2461; 1987.

171. Allen GP, Coogle LD. Characterization of an equine herpesvirus type 1 gene encoding a glycoprotein (gp 13) with homology to herpes simplex virus glycoprotein C. J of Virology 62:2850-2858; 1988.

172. Little SP, Joffre JT, Courtney RJ, Schaffer PA. A virion-associated glycoprotein essential for infectivity of herpes simplex type-1. Virology 115:149-160; 1981.

173. Blackslaws BA, Nash AA, Darby G. Specificity of the immune response of mice to herpes simplex virus glycoproteins B and D constitutively expressed on L cell lines. J Gen Virology 68:1103-1114; 1987.

174. Corey L, Spear PG. Infection with herpes simplex viruses. N Eng J Med 314:686-691; 1986.

175. Ostlund EN, Powell DG, Bryans JT. Equine herpesvirus-1: A Review. Proceedings Am Assoc Equine Pract. 1990.

176. Whitwell KE, Gower SM, Smith KC. An immunoperoxidase method applied to the diagnosis of equine herpesvirus abortion, using conventional and rapid microwave techniques. Equine Vet J 24:10-12; 1992.

177. Doll ER, Kintner JH. A comparative study of the equine abortion virus and equine influenza. Cornell Vet 44:355-367; 1954.

178. Burrows R, Goodridge D. Experimental studies on equine herpesvirus type 1 infections. J Reprod Fert, Suppl 23:611-615; 1975.

179. Couignoul FL, Bertram TA, Cheville NF. Pathogenicity of equine herpesvirus 1 subtype 2 for foals and adult pony mares. Vet Microb 9:533-542; 1984.

180. Comeny M. De la paraplegie infectieuse du cheval. Rec Med Vet 5:288-295; 1888.

181. Saxegaard F. Isolation and identification of equine rhinopneumonitis virus (equine abortion virus) from cases of abortion and paralysis. Nord Vet Med 18:504-516; 1966.

182. Pursell AR, Snagster LT, Byars TD, et al. Neurologic disease induced by equine herpesvirus 1. J Am Vet Med Assoc 175:473-474; 1979.

183. Meningoencephalomyelitis in horses associated with equine herpesvirus 1 infection. Vet Pathol 13:59-68; 1976.

184. Platt H, Singh H, Whitwell KE. Pathologic observation on an outbreak of paralysis in broodmares. Equine Vet J 12:118-126; 1980.

185. Edington N, Bridges CG, Patel JR. Endothelial cell infection and thrombosis in paralysis caused by equid herpesvirus-1: Equine stroke. Arch Virol 90:111-124; 1986.

186. Greenwood RES, Simon ARB. Clinical report of a paralytic syndrome affecting stallions, mares and foals on a Thoroughbred studfarm. Equine Vet J 12:113-117; 1980.

187. Gentry PA, Feldman BF, O'Neill SL, et al. Evaluation of the haemostatic profile in the pre- and post parturient mare, with particular focus on the perinatal period. Equine Vet J 24:33-36; 1992.

188. Whitwell KE, Blunden AS. Pathologic findings in horses dying during an outbreak of the paralytic form of Equid herpesvirus type 1 (EHV-1) infection. Equine Vet J 24:13-19; 1992.

189. Mumford JA, Rossdale PD, Jesset DM, et al. Serological and virological investigations of an equine herpesvirus-1 (EHV-1) abortion storm at a stud farm in 1985. J Reprod Fert, Suppl 35: 509-518; 1987.

190. Bryans JT. On immunity to disease caused by equine herpesvirus-1. J Am Vet Med Assoc 155: 294-300; 1969.

191. Edington N, Smyth B, Griffiths L. The role of endothelial cell infection in the endometrium, placenta and foetus of equid herpesvirus-1 (EHV-1) abortions. J Comp Pathol 104:379-387; 1991.

192. Coggins L. Viral respiratory of horses. Vet Clins N Am, Large Animal 1:59-72; 1979.

193. Thomson GR, Mumford JA, Campbell J, et al. Serological detection of equid herpesvirus 1 infections of the respiratory tract. Equine Vet J 8:58-65; 1976.

194. Gradil C, Joo HS. A Radial Immunodiffusion enzyme assay for detection of antibody to equine rhinopneumonitis virus (EHV-1) in horse serum. Vet Microb 17:315-322; 1988.

195. Dutta SK, Talbot NC, Myrup AC. Detection of equine herpesvirus-1 antigen and the specific antibody by enzyme-linked immunosorbent assay. Am J Vet Res 44:1930-1934; 1983.

196. Whitwell KE, Gower SM, Smith KC. An immunoperoxidase method applied to the diagnosis of equine herpesvirus abortion, using conventional and rapid microwave techniques. Equine Vet J 24:10-12; 1992.

197. Sharma PC, Cullinane AA, Onions DE, Nicoloson L. Diagnosis of equid herpesvirus -1 and -4 by polymerase chain reaction. Equine Vet J 24:20-25; 1992.

198. O'Keefe JS, Murray A, Wilks CR, Moriarty KM. Amplification and differentiation of the DNA of an abortogenic (type 1) and respiratory (type 4) strain of equine herpes virus by the polymerase chain reaction. Res Vet Sci 50:349-351; 1991.

199. Ahmed R, Stevens JG. Viral persistence. Virology, 2nd Edition, Fields RN, Knipe DM., Raven Press, New York, NY:241-265; 1990.

200. Burrows R, Goodridge R. Studies of persistent and latent infections in the Piribright pony herd. Latent Infections in Veterinary Medicine, Wittman G, Nijhoff, Boston, MA:307319; 1984.

201. Edington N, Bridges CG, Huckle A. Experimental reactivation of equid herpesvirus 1 (EHV 1) following administration of corticosteriods. Equine Vet J 17:369-372; 1985.

202. Dutta SK. Myrup AC. Infectious center assay of intracellular virus and infective virus titers for equine mononuclear cells infected in vivo and in vitro with equine herpesviruses. Can J Comparative Med 47:64-69; 1983.

203. Croen KD. Latency of the human herpesviruses. Ann Rev Med 42:61-67; 1991.

204. Doll ER, Bryans JT. Immunization against viral rhinopneumonitis of horses with live virus propagated in hamsters. J Am Vet Med Assoc 139:1324-1330; 1963.

205. Doll ER, Bryans JT. Immunization of young horses against viral rhinopneumonitis. Cornell Vet 53:24-41; 1963.

206. Lui KM, Castleman W. Equine posterior paresis associated with equine herpesvirus 1 vaccine in California: A preliminary report. J Eq Med Surg 1:397-401.

207. Burki F, Rossmanith W, Nowotny N, et al. Viraemia and abortions are not prevented by two commercial Equine Herpesvirus-1 vaccines after experimental challenge of horses. Vet Quarterly 12:80-86; 1990.

208. Cornick J, Martens J, Martens R, et al. Safety and efficacy of a thymidine kinase negative equine herpesvirus-1 vaccine in young horses. Can J Vet Res 54:26-266; 1990.

209. Bryans JT. On immunity to disease caused by equine herpesvirus 1. J Am Vet Med Assoc 155:294-300; 1969.

210. Fitzpatrick DR, Studdert MJ. Immunologic relationships between equine herpesvirus type 1 (equine abortion virus) and type 4 (equine rhinopneumonitis virus). Am J Vet Res 45:1947-1952; 1984.

211. Doll ER, Bryans JT. Development of complement-fixing and virus-neutralizing antibodies in viral rhinopneumonitis of horses. Am J Vet Res 23:843-846; 1962.

212. Thomson GR, Mumford JA, Campbell J, et al. Serological detection of equid herpesvirus 1 infections of the respiratory tract. Equine Vet J 8:58-65; 1976.

213. Gradil C, Joo HS. A radial immunodiffusion assay for detection of antibody to equine rhinopneumonitis virus-1 (EHV-1) in horse serum. Vet Microbiol 17:315-322; 1988.

214. Mumford JA, Rossdale PD, Jessett DM, et al. Serologic and virologic investigations of an equid herpesvirus-1 (EHV-1) abortion storm on a stud farm in 1985. J Repro Fert suppl 35:509-518; 1987.

215. Burki F, Rossmanith W, Nowotny N, et al. Viraemia and abortions are not prevented by two commercial Equine Herpesvirus-1 vaccines after experimental challenge of horses. Vet Quarterly 12:80-86, 1990.

216. Yewdell JW, Bennick JR. The binary logic of antigen processing and presentation to T cells. Cell 62:203-206; 1990.

217. Adler WH, Takiguchi T, Marsh B, Smith RT. Cellular recognition by mouse lymphocytes in vitro. I. Definition of a new technique and results of stimulation by PHA and specific antigens. J Exp Med 131:1049-1078;1970.

218. Lazary S, Dubath ML, Luder CH, Gerber H. Equine leukocyte antigen. Serologic and mixed lymphocyte reactivity studies in families. Transplantation 30:210-215;1980

219. Wilks CR, Coggins L. Immunity to equine herpesvirus type 1 (rhinopneumonitis) in vitro lymphocyte response. Am J Vet Res 37: 487-492; 1976.

220. Dupont B, Hansen JA. Human mixed-lymphocyte culture reaction: Genetics, specificity and biological implications. Adv Immunol 23:107-202; 1976.

221. Termijtelen A, van Leeuwen A, van Rood JJ. HLA-linked lymphocyte activating determinants. Immunol Rev 66:79-101;1982.

222. Ely JM, Prystowsky MB, Eisenberg L, et al. Alloreactive cloned T cell lines V. Differential kinetics of IL-2 CSF, and BCSF release by a cloned T amplifier cell and its variant. J Immunol 127:2345-2349; 1981.

223. Kirchner H, Zanatzky R, Engler H, et al. Production of interferon in the murine mixed lymphocyte culture. II. Interferon production is a T cell-dependent function, independent of proliferation. Eur J Immunol 9:824-826;1979.

224. O'Brien MA, Holmes MA, Lunn DP, Duffus WPH. Evidence for MHC class-I restricted cytotoxicity in the one-way, primary mixed lymphocyte reaction. Equine Vet J Suppl 12:30-34; 1991.

225. Rouse BT, Norley S, Martin S. Antiviral cytotoxic T lymphocyte induction and vaccination. Rev Inf Dis 10:16-33; 1988.

226. Thiele DL, Lipsky PE. The role of cell surface recognition in the initiation of MHC-unrestricted 'promiscuous' killing by T cells. Immunol Today 10:375-380; 1989.

227. Herberman RB. Natural killer cells. Natural Immunity, Nelson DS, Academic Press New York, NY: 71-122; 1989.

228. Hersey P. Non-MHC-restricted cytotoxic T lymphocytes. Natural Immunity, Nelson DS, Academic Press, New York, NY:123-146; 1989.

229. Lopez C, Kirkpatrick D, Read SE, et al. Correlation between low natural killing of fibroblasts infected with herpes simplex virus type 1 and susceptibility to herpesvirus infections. J Inf Dis 147:1030-1035; 1983.

230. Anderson K, Goodpasture EW. Infection of newborn Syrian hamsters with the virus of mare abortion. Am J Pathol 18:555-559; 1942.

231. Doll ER, Richard MG, Wallace ME. Adaption of the equine abortion virus to suckling Syrian hamsters. Cornell Vet 43:551-558; 1953.

232. Doll ER, Bryans JT, McCollum WH, Crowe EW. Propagation of equine abortion virus in Syrian hamsters. Cornell Vet 46:68-82; 1956.

233. Stokes A, Allen GP, Pullen A, Murray K. A hamster model of equine herpesvirus type 1 (EHV-1) infection; passive protection by monoclonal antibodies to EHV-1 glycoproteins 13, 14 and 17/18. J Gen Virol 70:1173-1183; 1989.

234. Rollinson EA, White G. Relative activities of acyclovir and BW759 against Aujeszky's disease and equine rhinopneumonitis viruses. Antimicrob Agents Chemother 24:221-226; 1983.

235. Patel JR, Edington N. The pathogenicity in mice of respiratory, abortion and paresis isolates of equine herpesvirus-1. Vet Micro 8:301-305; 1983.

236. Awan AR, Chong YC, Field HJ. The pathogenesis of equine herpesvirus type 1 in the mouse: a new model for studying host responses to the infection. J Gen Virol 71:1131-1140; 1990.

237. Awan AR, Gibson JS, Field HJ. A murine model for studying EHV-1-induced abortion. Res Vet Sci 51:94-99; 1991.

238. Kendrick JW, Stevenson W. Immunity to equine herpesvirus 1 infection in foals during the first year of life. J Repro Fert Suppl 27:615-618; 1979.

239. Frymus T. Humoral and cell-mediated immune response of foals vaccinated with attenuated equine herpesvirus type 1 (EHV-1). Zentralbl Veterinarimed 27:742-758; 1980.

240. Neeley DP, Hawkins DL. A two-year study of the clinical and serologic responses of horses to a modified live-virus vaccine. J Equine Med Surg 2:532-540; 1978.

241. Doll ER, Bryans JT. Development of complement-fixing and virus-neutralizing antibodies in viral rhinopneumonitis of horses. Am J Vet Res 23:843-846; 1962.

242. Bagust TJ, Pascoe RR. Studies on equine herpesviruses II. Development of a test antigen of cell culture origin for equine herpes-1 (rhinopneumonitis) virus and use in a microtitre system. Arch Virusforsch 36:240-250; 1972.

243. Shimizu T, Ishizaki R, Matumoto M. Two distinct antigens, viral and soluble of equine rhinopneumonitis virus as revealed by complement fixation test. Natl Inst An Health Quat 10:123-128; 1970.

244. Thomson GR, Mumford JA, Campbell J, et al. Serologic detection of equid herpesvirus 1 infections of the respiratory tract. Equine Vet J 8:58-65; 1976.

245. Edington N, Bridges CG. One way protection between equid herpesvirus 1 and 4 in vivo. Res Vet Sci 48:235-239; 1990.

246. Shimizu M, Satou K, Nishioka N. Monoclonal antibodies with neutralizing activity to equine herpesvirus 1. Arch Virol 104:169-174; 1989.

247. Guo P, Goebel S, Davis S, et al. Expression in recombinant vaccinia virus of the equine herpesvirus 1 gene encoding glycoprotein gp13 and protection of immunized animals. J Virol 63:4189-4198; 1989.

248. Guo P, Goebel S, Perkus ME, et al. Coexpression of vaccinia virus recombinants of equine herpesvirus 1 glycoproteins gp13 and gp14 results in potentiated immunity. J Virol 64:2399-2406; 1990.

249. Glorioso JV, Kees V, Hume H, et al. Identification of herpes simplex virus type 1 (HSV-1) glycoprotein C as the immunodominant antigen for HSV-1-specific cytotoxic T lymphocytes. J Immunol 135:575-582; 1985.

250. Rosenthal KL, Smiley S, South S, Johnson DC. Cells expressing herpes simplex glycoprotein gC but not gB, gD, or gE are recognized by murine virus-specific cytotoxic T lymphocytes. J Virol 61:2438-2447; 1984.

251. Kohl S, Starr SE, Oleske JM, et al. Human monocyte-macrophage-mediated antibody-dependent cellular cytotoxicity to herpes simplex virus-infected cells. J Immunol 118:729-735; 1977.

252. Shore SL, Melewicz FM, Gordon DS. The mononuclear cell in human blood which mediates antibody-dependent cellular cytotoxicity to virus-infected target cells. I. Identification of the population of effector cells. J Immunol 118:558-566; 1977.

253. Melewicz FM, Shore SL, Ades EW, Phillips DJ. The mononuclear cell in human blood which mediates antibody-dependent cellular cytotoxicity to virus-infected cells. II. Identification as a K cell. J Immunol 118:567-573; 1977.

254. Fujimiya Y, Perryman LE, Crawford TB.Leukocyte cytotoxicity in a persistent virus infection: presence of direct cytotoxicity but absence of antibody-dependent cellular cytotoxicity in horses infected with equine infectious anemia virus. Infect Immun 24:628-636; 1979.

255. Notkins AL. Immune mechanisms by which the spread of viral infection is stopped. Cell Immunol 11:478-483; 1974.

256. Wildy P, Bell PGH. The host response to herpes simplex virus. Br Bull Med 41:86-91; 1985.

257. Rouse BT, Horochov DW. Cytotoxic T lymphocytes in herpesviral infections. Vet Immunol Immunopathol 6:35-66; 1984.

258. Zingernagel RM, Doherty PC. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. Adv Immunol 27:51-177; 1979.

259. Zinkernagel RM, Rosenthal KL. Experiments and speculation on antiviral specificity of T and B cells. Immunol Rev 58:131-155; 1981.

260. Finberg R, Hom R. The role of T cell immunity in infection with herpes group viruses. Year Immunol 2:267-278; 1986.

261. Martin S, Rouse BT. The mechanisms of antiviral immunity induced by a vaccinia virus recombinant expressing herpes simplex type I glycoprotein D: clearance of local infection. J Immunol 138:3431-3437; 1987.

262. Martin S, Moss B, Berman PW, et al. The mechanisms of antiviral immunity induced by a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: cytotoxic T cells. J Virol 61:726-734; 1987.

263. Howes EL, Taylor W, Mitcheson NA, Simpson E. MHC matching shows that at least two T-cell subsets determine resistance to HSV. Nature (London) 277:66-68; 1979.

264. Pettinelli CB, Schmitt-Verhulst AM, Shearer GM. Cell types required for H-2 restricted cytotoxic responses generated by TNP-modified syngeneic cells or proteins. J Immunol 122:847-854; 1979.

265. Kolaitis G, Doymaz M, Rouse BT. Demonstration of MHC class II-restricted cytotoxic T lymphocytes in mice against Herpes simplex virus. Immunol 71:101-106; 1990.

266. Martin S, Cantin E, Rouse BT. Cytotoxic T lymphocytes: Their relevance in herpesvirus infections. NY Acad Sci.

267. Reddehase MJ, Koszinowski VH. Significance of herpesviral immediate early gene expression in cellular immunity to cytomegalovirus infection. Nature (London) 312:369-371; 1984.

268. Thomson GR, Mumford JA. In vitro stimulation of foal lymphocytes with equid herpesvirus 1. Res Vet Sci 22:347-352; 1977.

269. Gerber JD, Marron AE, Bass EP, Beckenhauer WH. Effect of age and pregnancy on the antibody responses of horses to equine herpesvirus 1. Can J Comp Med 41:471-478; 1977.

270. Wilks CR, Coggins L. Immunity to equine herpesvirus type 1 (rhinopneumonitis): In vitro lymphocyte response. Am J Vet Res 37:487-492; 1976.

271. Pachciarz JA, Bryans JT. Cellular immunity to equine rhinopneumonitis virus in pregnant mares. Proceedings Fourth International Conference Equine Infectious Diseases:115-127; 1978.

272. Dutta SK, Campbell DL. Cell mediated immunity in equine herpesvirus type 1 infection I. In vitro lymphocyte blastogenesis and serum neutralizing antibody in normal parturient and aborting mares. Can J Comp Med 41:404-408; 1977.

273. Lawman MJP, Naylor PT, Huang L, et al. Cell-mediated immunity to herpes simplex virus: induction of cytotoxic T lymphocyte responses by viral antigens incorporated into liposomes. J Immunol 126:304-308; 1981.

274. Lawman MJP, Courtney RJ, Eberle R, et al. Cell-mediated immunity to herpes simplex virus: specificity of cytotoxic T cells. Infect Immun 30:451-461; 1908.

275. Carpenter CB. Lymphocyte-mediated cytotoxicity. Manual of Clinical Laboratory Immunology, 3rd Edition, Rose NR, Friedman H, Fahey JL, American Society for Microbiology, Washington, DC:304-307; 1986.

276. Personal communication Dr. John Ortaldo, National Cancer Institute.

277. Edens LM. Unpublished results.

278. Wilks CR, Coggins L. In vitro cytotoxicity of serum and peripheral blood leukocytes for equine herpesvirus type 1-infected target cells. Am J Vet Res 38:117-121; 1977.

279. Krensky AM, Lanier LL, Engelman EG. Lymphocyte subsets and surface molecules in man. Clin Immunol Rev 4:95-138; 1985.

280. Widder KJ. Magnetic protein A microspheres: a rapid method for cell separation. Cli Immunol Immunopathol 14:395-400; 1979.

281. Molday RS, Yen RCK, Rembaum A. Application of magnetic microspheres in labelling and separation of cells. Nature 268:437-438; 1977.

282. Lunn DP, Holmes MA, Gibson J, et al. Haematological changes and equine lymphocyte subpopulation kinetics during primary infection and attempted re-infection of specific pathogen free foals with EHV-1. Equine Vet J Suppl 12:35-40; 1991.

283. Hodgin EC, McGuire TC, Perryman LE, Grant BD. Evaluation of delayed hypersensitivity responses in normal horses and immunodeficient foals. Am J Vet Res 44:1161-1167; 1978.

284. Darlington RW. The role of equine macrophages in resistance or susceptibility to infection with equine herpesvirus-1. Proceedings of the Fourth International Conference on Equine Infectious Diseases:129-140; 1978.

285. O'Callaghan DJ, Allen GP, Randall CC. Structure and replication of the equine herpesviruses. Proceedings of the Fourth International Conference on Equine Infectious Diseases:1-31; 1978.

286. Cunningham CH. Quantal and enumerative titration of virus in cell cultures. Tissue culture methods and applications, Academic Press, New York, NY; 527-535; 1973.

287. Schendel DJ, Wank R, duPont B. Standardization of the human in vitro cell-mediated lympholysis technique. Tissue Antigens 13:112-120; 1979.

288. Thomson GR, Mumford JA.In vitro stimulation of foal lymphocytes with equid herpesvirus 1.Res Vet Sci 22:347-352; 1977.

289. SAS user's guide: statistics, Version 6.03 edition. Cary, NC:SAS Institute Inc., 1988.

290. Tang JT, Delong DC, Butler LD, et al. Murine thymocytes mediate a natural killer like activity against herpes virus-infected target cells but not YAC-1 target cells. Scand J Immunol 24:115-118; 1986.

291. Kohl S, Loo LS. Ontogeny of murine cellular cytotoxicity to herpes simplex virus infected cells. Infect Immunol 30:847-850; 1980.

292. Loftin KC, Gonik B, Kumaran P. Natural killer cytotoxicity and antibody-dependent cell cytotoxicity to herpes simplex virus-infected target cells in murine pregnancy. Am J Repro Immunol Microbiol 17:53-56; 1988.

293. Darlington RW, James C. Biological and Morphological aspects of the growth of equine abortion virus. J Bacteriol 92:250-257;1966.

294. Esposito JJ, Murphy FA. Infectious recombinant vectored virus vaccines. Adv Vet Sci 33:195-247; 1989.

295. Edington N, Smyth B, Griffiths L. The role of endothelial cell infection in the endometrium, placenta and foetus of equid herpesvirus 1 (EHV-1) abortions. J Comp Pathol 104:379-387;1991.

296. Stutman O, Lattime EC, Figarella EF. Natural cytotoxic cells against solid tumors in mice: a comparison with natural killer cells. Fed Proc 40:2699-2704;1981.

297. Personal communication with Dr. JR Ortaldo, National Cancer Institute, Frederick, MD.

298. Edens LM. Unpublished results.

299. Rouse BT, Miller LS, Turtinen L, Moore RN. Augmentation of immunity to herpes simplex virus by in vivo administration of interleukin-2. J Immunol 134:926-930;1985.

300. Farrar JJ, Benjamin ML, Hilfiker M, et al. The biochemistry, biology and role of interleukin 2 in the induction of cytotoxic T cell and antibody forming B cell responses. Immunol Rev 63:129-166;1982.

301. Ortaldo JR. Lymphokine-activated killer cells: regulation of activity. In: Rees RC. The biology and clinical applications of interleukin-2. New York: IRL Press:221-234;1990.

302. Phillips JH, Lanier LL. Dissection of the lymphokine-activated killer phenomenon: Relative contribution of peripheral blood natural killers and T lymphocytes to cytolysis. J Exp Med 164:814-825;1986.

303. Ortaldo JR, Mason A, Overton R. Lymphokine-activated killer cells: Analysis of progenitors and effectors. J Exp Med 164:1193-1205;1986.

304. Merluzzi VJ. Comparison of murine lymphokine-activated killer cells, natural killer cells, and cytotoxic T lymphocytes. Cell Immunol 95:95-104;1985.

305. Feinberg BB, Tan NS, Walsh SW, et al. Progesterone and estradiol suppress human mononuclear cell cytotoxicity. J Repro Immunol 21:139-148;1992.

306. Vaquer S, De La Hera A, Jorda J, et al. Diminished natural killer activity in pregnancy: modulation by interleukin-2 and interferon gamma. Scand J Immunol 26:691-698;1987.

307. Lloyd S. Effect of pregnancy and lactation upon infection. Vet Immunol Immunopathol 4:153-176;1983.

308. Kuo YC, Lin CY. Recurrent herpes simplex virus type 1 infection precipitated by the impaired production of interleukin-2, alpha-interferon, and cell-mediated cytotoxicity. J Med Virol 31:183-189;1990.

309. Miller-Edge M, Splitter G. Patterns of bovine T cell-mediated immune responses to bovine herpesvirus 1. Vet Immunol Immunopathol 13:301-319;1986.

310. Harp JA, Kehrli ME, Hurley DJ, et al. Numbers and percent of T lymphocytes in bovine peripheral blood during the periparturient period. Vet Immunol Immunopathol 28:29-35;1991.

311. Iwatani Y, Amino N, Tachi J, et al. Changes of lymphocyte subsets in normal pregnant and postpartum women: postpartum increase of NK/K (Leu 7) cells. Am J Repro Immunol 18:52-55;1988.

312. Lunn DP, Holmes MA, Duffus WPH. Three monoclonal antibodies identifying antigens on all T lymphocytes, and two mutually exclusive T-lymphocyte subsets. Immunology 74:251-257;1991.

313. Crump AL, Davis WC, Antczak DF. A monoclonal antibody identifying a T cell marker in the horse. Anim Genet 19:349-357;1988.

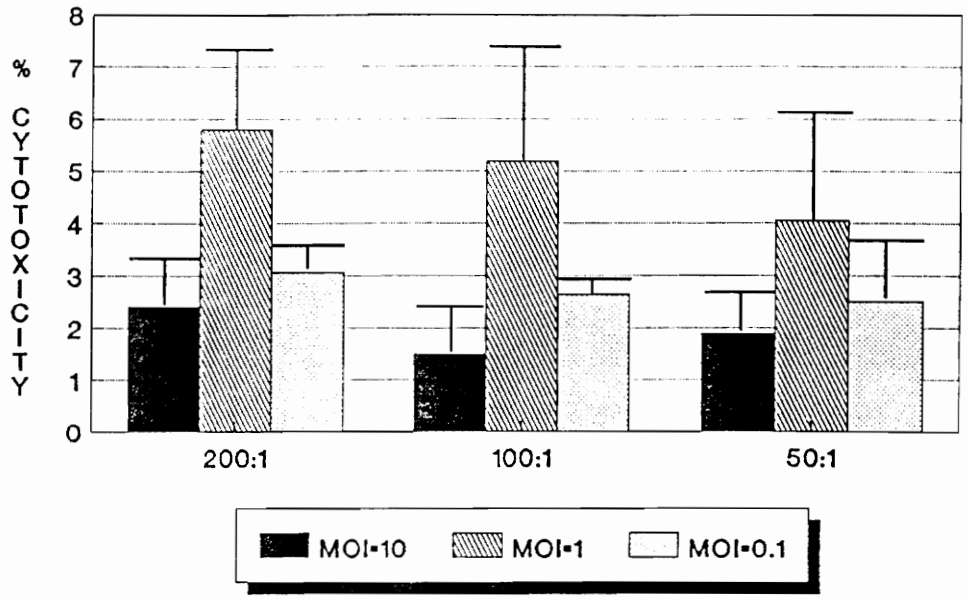


Figure 1. The effect of multiplicity of infection (MOI) on the percent cytotoxicity of non-adherent PBMC from 5 non-pregnant mares against EHV-1 infected EDF. Percent cytotoxicity is expressed as the mean $\pm$ the standard deviation.

Table 1. The effect of EHV-1 infection on the specificity of the cytotoxic response to EDF. Lymphocytes from 5 non-pregnant mares were used immediately after isolation or after a 72 hour in vitro incubation with IL-2. Percent cytotoxicity is expressed as the mean ± standard error for each of the 3 effector to target cell ratios (200:1, 100:1, and 50:1).

	% Cytotoxicity	
	Freshly Isolated	IL-2 Stimulated
EDF		
200:1	0.89 ± 0.90[a]	10.66 ± 3.47[c]
100:1	0.52 ± 1.13[a]	10.36 ± 4.72[c]
50:1	0.77 ± 1.01[a]	7.78 ± 4.39[c]
EHV-EDF		
200:1	4.68 ± 2.64[b]	16.76 ± 3.56[d]
100:1	4.66 ± 2.12[b]	18.22 ± 3.26[d]
50:1	3.24 ± 2.01[b]	13.14 ± 5.71[d]

a,b $p \leq 0.0582$ difference between the percent cytolysis of infected and uninfected EDF by freshly isolated lymphocytes. The percent cytotoxicity was reduced as the effector to target cell ratio was lowered.

c,d $p \leq 0.0002$ difference between the percent cytolysis of infected and uninfected EDF by IL-2 stimulated lymphocytes. There was no effect on the percent cytotoxic activity by altering effector to target cell ratios.

Table 2. The effect of pregnancy and 72 hours stimulation with IL-2 on the percent cytotoxicity against EHV-1 infected EDF by equine lymphocytes. Percent cytotoxicity is expressed as the mean ± standard deviation for each of the 3 effector to target cell ratios (200:1, 100:1, and 50:1).

	% Cytotoxic Activity Against EHV-1 infected EDF	
	Freshly Isolated	IL-2 Stimulated
Pregnant		
200:1	1.34 ± 1.16[ab]	25.10 ± 6.67[ef]
100:1	0.46 ± 0.97[ab]	26.52 ± 7.25[ef]
50:1	0.28 ± 0.47[ab]	22.72 ± 6.38[ef]
Non-Pregnant		
200:1	4.68 ± 2.64[cd]	16.76 ± 3.56[gh]
100:1	4.66 ± 2.12[cd]	18.22 ± 3.26[gh]
50:1	3.24 ± 2.01[cd]	13.14 ± 5.71[gh]

a,c $p \leq 0.02$ difference in the percent cytotoxicity from freshly isolated equine lymphocytes from pregnant mares when compared to non-pregnant mares.

b,f $p \leq 0.0002$ difference in the percent cytotoxicity between freshly isolated and IL-2 stimulated equine lymphocytes from pregnant mares.

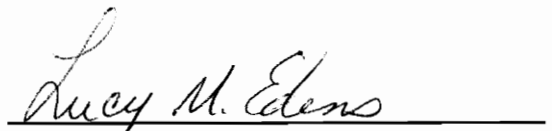
e,g $p \leq 0.03$ difference in the percent cytotoxicity from IL-2 stimulated equine lymphocytes from pregnant mares when compared to non-pregnant mares.

d,h $p \leq 0.0002$ difference in the percent cytotoxicity between IL-2 stimulated equine lymphocytes from non-pregnant mares.

VITA

Lucy M. Edens, D.V.M.

Dr. Lucy Edens was born on January 31, 1961 in Corpus Christi, Texas. She attended Texas A & M University receiving a B.S. in Biomedical Science in 1982, graduating Magna Cum Laude. After being accepted to the College of Veterinary Medicine at Texas A & M University she was awarded a B.S. in Veterinary Science (Cum Laude) in 1984 and her Doctor of Veterinary Medicine in 1986. Upon graduation she entered a mixed animal practice in southern California with her husband we they practiced together for 3 years. In 1989 she began a residency in large animal internal medicine at the Marion duPont Scott Equine Medical Center. During her residency she was also enrolled in a graduate program through the Virginia-Maryland College of Veterinary Medicine and Virginia Polytechnical Institute and State University. After completing the clinical portion of the program in 1992 she accepted a position as a clinical instructor in large animal medicine at the University of Florida, College of Veterinary Medicine. In 1993 she was awarded diplomate status in the American College of Veterinary Medicine and accepted a position as an assistant professor in large animal medicine at the University of Florida. At the present time she is pursing research on viral respiratory disease in horses.



Lucy M. Edens, D.V.M.
Diplomate ACVIM