Coignoul, Freddy Louis

FUNCTIONAL AND ULTRASTRUCTURAL CHANGES IN NEUTROPHILS IN NORMAL AND EQUINE HERPESVIRUS 1 SUBTYPE 2 INFECTED MARES AND FOALS

Iowa State University PH.D. 1983

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark ✓.

1. Glossy photographs or pages ✓
2. Colored illustrations, paper or print _____
3. Photographs with dark background ✓
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages _____
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Other _____

Functional and ultrastructural changes in neutrophils in normal and equine herpesvirus 1 subtype 2 infected mares and foals

by

Freddy Louis Coignoul

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

Approved:

Members of the Committee:

Signature was redacted for privacy.

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1983

TABLE OF CONTENTS

GENERAL INTRODUCTION

Equine herpesvirus 1 (EHV-1, rhinopneumonitis virus, equine abortion virus) is responsible for interstitial pneumonitis, necrotizing bronchiolitis, and upper respiratory disease in foals up to 1 year of age (1, 2). Foals infected in utero die of peracute generalized infection. Aborted fetuses have multiple necrotic foci in liver, lungs, and lymphoid organs. Intranuclear inclusion bodies are consistently present at the edge of necrotic lesions (2, 3). Adult horses are usually asymptomatic carriers of the virus. However, a neurologic syndrome characterized by transient paralysis has been described in adult horses (4, 5).

Two subtypes of EHV-1 exist, and are genetically and serologically distinct. Subtype 1 (abortigenic virus, subtype F), the most virulent, is responsible for systemic infections and can cause any form of the disease (6, 7). Subtype 2 (subtype R, respiratory virus) is only pathogenic for foals, provokes anterior respiratory infections (6), and has not been shown to spread systemically. Many foals have serum antibodies against EHV-1 without a history of clinical disease (7). The role of the 2 subtypes in inapparent infections has not been clearly established.

The immune response of foals and adult horses experimentally inoculated with EHV-1 is markedly different. Inoculated foals develop a cell mediated immune (CMI) response to the virus, as shown by lymphocyte blastogenesis (8, 9, 10) and leukocyte migration inhibition (11) assays.

However, in contrast with adult horses, foals rarely develop detectable levels of virus neutralizing (VN) antibodies after a single exposure to the virus (8, 11, 12). Cell mediated immunity has been shown to be the major protective mechanism against EHV-1 (9, 11, 13). Antibodies, on the other hand, are of short duration (14) and do not protect against reinfection (11). Since studies of lymphocytes and their products do not appear to adequately explain the susceptibility of foals to the virus, other components of the host defense mechanisms should be investigated.

Neutrophils are likely to play a major role in EHV-1 infections. Neutrophils can inactivate viruses directly (15) and lyse virus-infected cells by antibody dependent cell mediated cytotoxicity (ADCC). Neutrophils are particularly active against herpesvirus-infected cells, as demonstrated in humans (16) and cattle (17). In addition, efficiency of neutrophils in killing bacteria should also limit secondary bacterial infections responsible for life-threatening complications in EHV-1 infected foals (18, 19).

The present study was undertaken to: (i) characterize and compare ultrastructure and functions of peripheral blood neutrophils in normal foals and adult horses; (ii) compare neutrophil morphology and function in foals and adult horses experimentally infected with EHV-1 subtype 2; and (iii) report clinical, serologic, hematologic, and virologic characteristics of experimental infection with EHV-1 subtype 2 in foals and adult horses.

The dissertation is presented in alternate format including 3 manuscripts submitted for publication in scientific journals. The format used for the dissertation is that of the American Journal of Veterinary Research. A literature review precedes the first manuscript, and a general discussion follows the last manuscript. Literature cited is listed once at the end of the dissertation.

Freddy L. Coignoul is the principal investigator for each part of this study.

LITERATURE REVIEW

History of the neutrophil In 1884 in Virchows Archives, Elie Metchnikoff introduced a new theory of immunity: phagocytosis. At the time, the idea of bacterial digestion by body cells was severely criticized by the tenants of humoral immunity. By 1902, however, Leishman had quantitated bacterial uptake by human neutrophils and in 1903, Wright and Douglas demonstrated the importance of opsonization in phagocytosis (20).

These early studies became for decades the framework of research on phagocytosis and killing of bacteria by neutrophils. In vitro techniques were developed for neutrophil function studies. The Boyden chamber, composed of two compartments separated by a millipore filter, allowed evaluation of neutrophil migration and chemotaxis (21). Efficiency of phagocytosis and killing of ingested bacteria were accurately measured under various conditions (22). In particular, the increased consumption of oxygen and glucose associated with the bactericidal activity of neutrophils was demonstrated in vitro (23, 24). Accurate studies on dynamics of granulopoiesis and neutrophil lifespan were possible by incorporation of radio-labeled markers in granulocyte precursors (25, 26). Evaluation of detailed neutrophil structure and how it relates to function became possible with the use of the electron microscope coupled with histochemical procedures. In an excellent series of articles, Bainton and Farquhar demonstrated the existence of two distinct granule populations in rabbit neutrophils. Primary granules are lysosomes that

contain hydrolytic enzymes and antibacterial agents. They originate from the inner face of the Golgi complex at the progranulocyte stage (27, 28). Secondary granules develop later, and originate from the outer face of the Golgi complex of myelocytes. In the rabbit, primary granules contain hydrolases and secondary granules contain alkaline phosphatase (29). More recently, emphasis was placed on the role of cytoskeleton, especially microtubules and microfilaments (30, 31, 32), and on the role of regulatory mechanisms (33, 34) on neutrophil functions.

Neutrophil functions The major role of neutrophils is to clear microorganisms from injured tissues. Events associated with that function can be divided into three major steps: (i) margination and migration of circulating neutrophils through the vascular wall; (ii) phagocytosis; and (iii) killing of living microorganisms.

(i). Margination and migration. Complement fragment C5a (anaphylatoxin) is responsible for initiating margination by attaching to specific receptors of neutrophil membranes and increasing adhesiveness and causing aggregation of circulating neutrophils (35). Margination may also be due to modifications in the blood vessel endothelium (36). Glycocalyx modifications, calcium-dependent adhesion of leukocytes, and modifications of surface electrical charge have been suggested as possible modifications of endothelial cells during injury (36). Experimental evidence, however, has not been conclusive and the issue of endothelial involvement in margination of neutrophils is still controversial.

After margination, neutrophils migrate into tissues in response to chemoattractants released at the lesion site. Chemotactic factors include lymphokines; products of complement, coagulation, and kinin cascades; bacterial products; collagen; fibrin; and cell membrane oxidized lipids (35).

Chemotaxis has been extensively studied in vitro and N-formylated peptides have provided a useful tool in many species. In bacteria, protein synthesis starts with N-formylated methionine (f-Met). A specific transfer RNA brings f-Met to the ribosome to initiate protein synthesis. As a consequence, f-Met is commonly present at the N terminal of bacterial peptides (37). In eukaryotes, protein synthesis also starts with methionine at the N terminal but without a formyl group attached to the first amino acid. Neutrophils have specific receptors for N-formyl methionine oligopeptides (38).

Latex beads coated with serum will adhere to neutrophil membranes in presence of f-Met peptides. In response to a concentration gradient of the peptide, adherent neutrophils develop a polarized morphology with a "leading edge" of ruffled membrane. Latex beads on the cell surface move toward the posterior pole of the cell, suggesting redistribution of membrane receptors (39). Neutrophils can react to a 1% differential concentration gradients across a space the width of their own diameter by orienting themselves and moving toward the highest concentration area (40, 41).

Chemotaxis is a complex phenomenon involving membrane, cell organelles, and cytoskeleton. High voltage electron microscopy has

shown that in response to chemoattractants (N-formylated peptides and C5a opsonized zymosan), the microtubule organizing center (the centrosome) moves between the anterior lamellipodium and the nucleus. Ruffling of cell membrane occurs first at the leading edge and microtubules polymerize subsequently (42).

Cells migrate toward the chemical gradient as long as microtubules are polymerized. Agents that depolymerize microtubules or prevent their assembly impair chemotaxis but not random migration (30, 34, 41, 43). Agents that interfere with microtubule assembly also induce capping of receptors on the cell surface (32, 44), indicating that microtubules also regulate distribution of receptors on the cell membrane. Microtubule polymerization is increased by magnesium and guanosine triphosphate (GTP). Tubulin dimer polymerization requires GTP (45). Free tubulin dimers are in equilibrium with polymerized tubulin. On the other hand, calcium and microtubule inhibitors such as colchicine, benzimidazole, or carbamates promote depolymerization by increasing the critical free tubulin concentration required for microtubule assembly (45).

In order for the neutrophil to react to and move toward chemotactic gradients, free receptor sites must be constantly available at the leading edge of the cell. During chemotaxis, secondary granules located at the anterior pole of the neutrophil spontaneously release their content (46). The membrane of the granules fuses with the cell membrane and it is believed that by that mechanism new free receptors are externalized at the anterior pole of the cell (41). Secondary granule

products can activate the complement system and generate chemotactic factors including C5a. On the other hand, proteases contained in primary granules can inactivate chemotactic factors, providing the neutrophil with mechanisms for both amplification and limitation of the inflammatory response (41).

(ii). Phagocytosis. At the site of injury, Fc and C3b receptors on the neutrophil mediate adhesion to antibody and complement coated particles (47). Internalization of particles is initiated by microfilaments and polymerization of actin is driven by ATP and calcium (45). Although it is not established if triggering of Fc and C3b receptors is different from stimulation leading to migration, phagocytosis is characteristically accompanied by an intracellular increase in calcium and cyclic AMP (48, 49). This increase is responsible for microfilaments polymerization and microtubule disassembly (45). Phagocytosis is completed by internalization of the particle and formation of the phagosome (47).

(iii). Killing. Upon recognition of the phagocytic stimulus, a series of biochemical events occur in neutrophils. There are a 2-20 fold increase in oxygen consumption and an increase in glucose metabolism via hexose monophosphate pathway (HMP) (50). The enzyme system responsible for oxygen consumption is a nicotinamide adenine dinucleotide (NADPH) dependent oxidase located in the plasma membrane and in the phagolysosome membrane (50). Superoxide anion (O_2^-), an activated oxygen radical, and hydrogen peroxide (H_2O_2) are produced upon stimulation. These toxic derivatives kill bacteria, fungi, tumor cells, and parasites

(49). Hydrogen peroxide also serves as a substrate for myeloperoxidase, an enzyme located in primary granules and liberated by phagolysosome fusion (48). Hydrogen peroxide is bactericidal by itself, but microbicidal activity is increased 50 times in the presence of myeloperoxidase and a halide (50). Enhanced activity is due to the formation of a H_2O_2-myeloperoxidase-halide complex capable of halogenation and oxidation of the microorganism surface (23). In addition, the complex can also decarboxylate amino acids and generate toxic aldehydes, ammonia, and CO_2 (50).

Methods have been developed that evaluate the oxidative burst of the neutrophil (23, 51). Superoxide anion can reduce nitroblue tetrazolium (NBT) and cytochrome C or oxidize epinephrine to adrenochrome. For methodology, see Fantone and Ward (50).

Additional highly reactive metabolites are also produced during neutrophil activation. Hydroxyl radicals (OH^-) are produced by interaction of H_2O_2 and O_2^- but the exact mechanism of production is still controversial (50). The reaction of two superoxide anion molecules results in generation of singlet oxygen, a strongly electrophilic molecule. Two species of singlet oxygen, sigma (Σ^1O_2) and delta (Δ^1O_2) are produced, due to an inversion of their electron spin (50). Production of singlet oxygen is due to superoxide dismutase, that splits the superoxide anion into two singlet oxygen ions (50). Under certain conditions, chemiluminescence results from interaction of singlet oxygen with another molecule. This characteristic reaction has been used in <u>in</u>

vitro assays of oxygen metabolism in the neutrophil (50). The real importance of different radicals *in vivo* is not well-established.

Generation of NADPH through the hexose monophosphate pathway is necessary for the activity of membrane oxidase. In the rare genetic human disease glucose 6-P dehydrogenase deficiency, decreased NADPH production results in loss of substrate for the oxidase and decreased respiratory burst (50). Hydrogen peroxide released in the phagosome and in the environment may diffuse into the neutrophil cytoplasm. A catalase and a peroxidase present in the cell cytoplasm detoxify H_2O_2 into water and oxygen. The peroxidase reduces glutathione. Reduced glutathione is regenerated by a glutathione reductase, with NADPH as the reducing agent (50).

Non-oxygen-dependent killing mechanisms of neutrophils have been much less studied than the oxygen-dependent mechanisms. Enzymes responsible for non-oxidative killing are mostly located in secondary granules. Enzyme distribution between primary and secondary granules varies extensively among species. It is generally accepted, however, that primary granules are lysosomes containing hydrolytic enzymes and peroxidase (29, 52). Secondary granules are secretory granules that contain lysozyme, lactoferrin, and vitamin B_{12} binding protein (46, 49, 52).

Lactoferrin is an iron binding protein that traps free iron necessary for growth of bacteria in inflammatory foci. An interesting concept has emerged recently on the possible role of lactoferrin in granulopoiesis regulation (53). Monomeric lactoferrin released by

specific granules would completely inhibit the secretion of a monocyte product that recruits T lymphocytes to secrete "colony stimulating activity" (CSA) factor. During inflammation, lactoferrin is released by neutrophils and polymerizes in a concentration-dependent fashion. Lactoferrin polymers do not inhibit CSA production by T cells (53), thus allowing granulopoiesis to increase. Although still largely unproven in vivo, this theory, if correct, may be the answer to granulopoiesis regulation that is still a mystery.

Phagocytized microorganisms are first exposed to the secondary granule's content, as secondary granules are first to degranulate into the phagosome. Later, primary granules contact the phagosome.

Microorganisms have developed various mechanisms to survive within neutrophil phagosomes (47). As a consequence of hydrolytic enzyme release from neutrophil granules, thromboxanes, endoperoxides, hydroxy and hydroperoxy acids, and leukotrienes are produced by oxidation of membrane lipids (49). Thromboxane and endoperoxides are transformed to prostaglandins which raise the cAMP levels in the cell, depolymerize microtubules, and decrease further degranulation (49). Hydroxy and hydroperoxy acids along with leukotrienes exert potent effects on blood vessels and blood cells (54). The release of these substances by granulocytes is now under investigation and should lead to a better understanding of the role of the neutrophil in the inflammatory process.

Role of neutrophils in viral diseases For a long time, neutralization of viruses has been solely associated with antibodies and complement (55). Studies of relations between leukocytes and viruses

were limited to modifications of white blood cell counts during viral infections (56) and recovery of infectious agents from blood fractions (57). Influenza virus was found to adhere on leukocyte surface, maybe through specific receptors (56). Phagocytosis of herpes simplex virus by neutrophils was demonstrated by Jamini in 1937 (56), but there was controversy about the fate of internalized viruses.

Viruses can be transported by leukocytes in the blood but the role of the neutrophil in that function is not clear for many viruses. Infectious bovine rhinotracheitis (IBR) virus is carried in the blood adsorbed on the surface of leukocytes (58). Equine herpesvirus 1, on the other hand, is transported intracellularly, where it can be isolated even in presence of circulating neutralizing antibodies (58).

The neutrophil myeloperoxidase-hydrogen peroxide-halide complex is a potent virucidal system against polio and vaccinia viruses (15). Production of interferon by neutrophils has been disregarded in the early days, and the term "leukocyte interferon" was coined to characterize monocyte products (56). In 1980, Rouse, Babiuk, and Henson reported that exposure of bovine neutrophils to inactivated IBR virus induced the release of interferon (59). This research team is the only one in the current literature to associate neutrophils with interferon production.

Antibody dependent cell mediated cytotoxicity (ADCC) refers to the ability of some blood and tissue cells, called effector cells, to attach to antibody coated target cells, and mediate cell lysis. Null cells, B lymphocytes, blood monocytes, neutrophils, and eosinophils can

lyse virus-infected cells coated with antibodies (60). Efficiency of different cell types varies with species, nature of the target cell, and experimental conditions.

The discovery by Gale in 1975 (61), that neutrophils could be effector cells in ADCC, was responsible for a renewed interest in the role of neutrophils in viral infections. Antibody dependent cell mediated cytotoxicity has been reported with neutrophils of rabbit (60), human (16), bovine (17), and murine (62) origin. In cattle, neutrophils are the most efficient mediators of ADCC against IBR virus-infected cells (63).

Target cells have a passive role in ADCC, the only requirement is that they are coated with antibodies. Therefore, virus infected cells, heterologous erythrocytes, and antibody coated tumor cells can be used with success as target cells. Cells infected with herpesviruses have been most often utilized. During replication of herpesviruses, viral proteins are inserted in the cell membrane, as early as 3 hours after infection (16). Antibodies attach to viral antigens in the cell membrane and are recognized by Fc receptors of neutrophils. Many scientists believe that ADCC is an *in vitro* demonstration of a cell mediated host defense mechanism that may take place *in vivo* during herpesvirus infection (16, 17, 64, 65). However, there is no direct evidence that ADCC occurs in tissues.

Confusion also exists on the mechanism of cell lysis by effector cells. The cytotoxic reaction requires energy, divalent cations, and intact microtubules and microfilaments (66). It is independent from

nucleic acid and protein synthesis (66). In an excellent paper, Grewal, Carpio, and Babiuk reported ultrastructural studies of ADCC of herpesvirus infected cells by bovine neutrophils (67). Attachment of neutrophils to target cells occurred 30 minutes after exposure, with formation of rosettes around the infected cells. Strong contact between cell membranes occurred after 2 hours exposure and as incubation continued neutrophil pseudopods surrounded the infected cell and penetrated into the cytoplasm. Target cells became increasingly swollen, with cytoplasmic vacuolation and nuclear membrane distortion. Ultimately, cell lysis occurred. Surprisingly, degranulation of neutrophils did not take place before 6 to 8 hours after exposure. The authors suggested a membrane to membrane interaction, maybe with formation of transmembrane channels (67). Another aspect of ADCC also mentioned in the same article (67) is that tightly packed neutrophils may form a continuous wall around the target cell, hindering virus from spreading to other susceptible cells.

Complement is not needed for ADCC. Complement-dependent, neutrophil-mediated cytotoxicity (CDNC), different from ADCC (and independent from antibodies), has been proposed (68) but to date has not been documented in the literature.

Studies on equine neutrophils Ultrastructurally, granules of equine neutrophils are polymorphic. Primary granules are larger than secondary granules and are round to ovoid with a dense granular matrix (69). In addition to primary and secondary granules common to all species, granules with a flocculent matrix are consistently present.

Flocculent granules are considered to be partially degranulated primary granules (70). Granules with a crystalline array in the matrix are occasionally seen in horse neutrophils. Similar structures have been described in man, where they are considered as immature secondary granules (70).

Functionally, equine neutrophils resemble those of other species, as shown by in vitro studies. Phagocytosis and killing have been studied in the presence of equine serum (71) and genital secretions (72). Chemiluminescence is produced after exposure to glass beads (73) and chemotaxis is reported to be very weak in response to f-Met peptides (74). Equine neutrophils fail to mediate ADCC with equine infectious anemia (EIA) virus infected cells (75). It has been suggested that IgG(T), a subclass of immunoglobulin G present in equine serum, coats virus infected cells with high affinity but does not react with the Fc receptors of the neutrophils (75). Other observations show that IgG(T) also interferes with complement fixation tests against EIA virus by competition with IgG (76).

History of equine herpesvirus 1 infection Abortion in mares was first suspected to be caused by a virus in 1922 when an outbreak of 11 abortions occurred at the Kentucky Agricultural Experimental Station (77). The first report of a virus causing equine abortion appeared in 1933 (78). The virus was not isolated but the distinctive lesions of the disease in the foal were described by Dimock in 1936 (77). Intracellular inclusion bodies were seen in hepatocytes and pneumocytes.

Tissues were submitted to Goodpasture who "expressed the opinion that the intranuclear inclusions are typically viral in nature" (79).

In 1941, Manninger and Czontos noticed the relation between abortion and respiratory disease. They described respiratory signs in a horse inoculated with material recovered from an aborted fetus and concluded that abortion results from infection of pregnant mares with equine influenza virus (80). The lesions described in the fetus indicate that the virus was EHV-1. Doll, in 1953, induced abortion in 10 mares after intrauterine inoculation with "the abortion virus" (81). Lesions seen in the foals were similar to descriptions of Dimock and Edwards (77), and Manninger and Czontos (80). In 1957, Doll et al. demonstrated that equine arteritis and equine abortion were 2 separate diseases caused by distinct viruses but both were responsible for respiratory signs and abortion in horses (82). From then, and upon their suggestion, the disease caused by "equine abortion virus" was referred to as equine rhinopneumonitis; the agent was called equine rhinopneumonitis virus (ERP virus).

First evidence that ERP virus is a herpesvirus came from the work of Plummer and Waterson in 1963 (83). They compared ERP with a known herpesvirus that they had isolated from the respiratory tract of a horse. Although the 2 viruses were morphologically similar, they differed in several other properties. Classification of both agents in the herpesvirus group was proposed. The new virus was called equine herpesvirus 2. ERP virus, serologically different, became equine

herpesvirus 1 or EHV-1. A third equine herpesvirus (EHV-3) was discovered later (84).

Recently, it was suggested that only some strains of EHV-1 were responsible for abortion (84, 85). Restriction endonuclease studies of DNA patterns confirmed the differences observed between abortigenic (subtype 1) and respiratory (subtype 2) strains of EHV-1. Literature prior to endonuclease studies is often confusing because in most cases, there is no clear reference to the strain of virus used.

Some isolates of EHV-1 subtype 1 are responsible for central nervous system disease in horses (3, 4). The question of whether that property corresponds to differences in virus genome is still under investigation at the present time (6).

Pathogenesis of equine herpesvirus 1 infection Infection with EHV-1 is thought to occur by inhalation of virus from horses with overt clinical signs. The virus attaches to and replicates in the anterior respiratory tract epithelium (6). Abortigenic EHV-1 infects lymphocytes and is transported in the blood; there is no documented evidence of viremia with EHV-1 subtype 2 (6). Equine herpes 1, like many other herpesviruses, probably attaches to target cells by adsorption (86). There is no evidence of specific receptors on the cell membrane and unenveloped particles attach as readily as virions (87). Phagocytosis has been proposed as the major mechanism of penetration (88). A possible alternate mechanism of penetration is the fusion of the virion envelope with the cell membrane and release of uncoated particles in the cytoplasm (87, 88).

In primary infection, virus is present in the nasal cavity for up to 10 days and disappears when neutralizing antibodies develop (5). Virus can be isolated from white blood cells for at least 24 days after infection (5). It is believed that EHV-1 is carried intracellularly in leukocytes where it is protected from circulating antibodies (58, 89). This concept of cell associated viremia has been used to explain abortion in immune pregnant mares (3, 9). Pathogenesis of pneumonia induced by respiratory strains has not been documented. Experimental aerosolization can induce pneumonia, it is logical to consider that viremia is not required. Airborne viruses can infect the lung directly or after replication in the anterior respiratory tract.

Pathology of equine herpesvirus 1 infection Pathogenicity of EHV-1 is restricted to horses and can be expressed in three types of clinical syndrome: abortion, pneumonia, and neurologic disease.

1. In pregnant mares, EHV-1 is responsible for abortions. Lesions in fetuses have been reported by Prickett (2). In rare cases where abortion occurs before 6 months of fetal age, autolytic fetuses are expelled with their membranes. Foci of necrosis and intranuclear inclusion bodies are present in many tissues, liver and spleen being most often affected (2). Abortions occur most frequently after the 7th month of gestation. Aborted fetuses are found dead, with petechiae on mucous membranes; jaundice; subcutaneous, pulmonary, and pleural edema; splenic enlargement; and multiple foci of necrosis in the liver (2).

Microscopically, multifocal liver necrosis, bronchiolitis, interstitial pneumonitis, and severe necrosis of the white pulp of the spleen are associated with typical herpetic intranuclear inclusion bodies (80).

Perinatal mortality has been reported in foals, probably from infections acquired in utero (5). The most prominent lesions are interstitial pneumonia and extensive necrosis in thymus and spleen (3). Mares aborting from EHV-1 infection do not have other clinical signs but the virus can be recovered from blood leukocytes and various tissues.

2. Foals infected experimentally by aerosol exposure have multiple tiny plum-colored foci in apical and cardiac lobes of the lungs. The mucosa of the nasal septum and proximal trachea is ulcerated. When horses are inoculated with EHV-1 via a nasal spray, there is epithelial hyperplasia of the nasal cavity and trachea; no gross lesion is seen in deeper parts of the respiratory tract (2).

Fetuses and newborn foals infected in utero have diffuse or multifocal purple lung lesions and intense lung edema with froth in the respiratory airways (1, 5). Submucosal hemorrhages are found along the trachea and bronchi and bronchial lymph nodes are often enlarged (1, 5). Areas of consolidation in the apical areas of the lung of adult horses have also been seen (82).

Microscopic lesions, without the complication of secondary bacterial infections, are characterized by epithelial necrosis, histiolymphocytic infiltration, and intranuclear inclusion bodies of the anterior respiratory tract mucosa (2). Lung changes, in both stillborn and neonatal foals include atelectasis, severe alveolar and bronchial edema with

fibrin casts and histiocytic alveolitis (2), mild to severe acute focal necrotizing bronchitis, bronchiolitis, and alveolitis with intranuclear inclusion bodies in bronchiolar epithelial cells (1, 2, 5, 19).

Often, hemolytic streptococci, Pasteurella multocida, and Escherichia coli are responsible for secondary acute purulent bronchopneumonia (18, 19). Lesions include massive neutrophilic infiltration of bronchi and bronchioles and serofibrinous exudation. Necrosis of bronchial epithelium and alveoli, with typical intranuclear inclusion bodies at the edges of the necrotic areas. Peribronchiolar and perivascular infiltration by macrophages and lymphocytes are described (2, 3). Ten days after inoculation of young horses, there is hyperplasia of bronchiolar epithelium, epithelialization of the alveolar lining, and intense infiltration of lymphocytes around bronchioles and pulmonary blood vessels. Bronchiole-associated lymphoid tissue is hyperplastic but no inclusion bodies are present in the lesions (2).

3. A neurologic syndrome has been reported with EHV-1 in both pregnant (4), and barren mares, stallions, and foals (5, 6). Vascular changes associated with neuropile degeneration in brain and spinal cord have been documented (4). Virus has been identified in blood vessel endothelial cells of the central nervous system (6).

Immunity against equine herpesvirus 1 Foals experimentally inoculated with EHV-1 develop moderate levels of neutralizing and complement fixing antibodies in the blood 1 to 3 days after exposure (90). Circulating antibodies do not protect horses against reinfection (9). However, high antibody titers seem to serve as a barrier against general-

ization of the disease and pregnant mares with high antibody titers appear to be protected against abortion (3).

In the respiratory tract, IgA antibodies are probably primarily involved in protection, since pneumonia can occur without viremia. Resistance to infection lasts only 3 to 4 months (90), consequently the role of antibodies is probably of only limited significance in providing an efficient protection.

Cell mediated immunity (CMI) is of paramount importance in herpesvirus infections (8, 9, 90). Horses infected experimentally with EHV-1 develop both humoral and cell mediated immunity (9). Studies of CMI based on lymphocyte blastogenesis assays have given erratic results. According to Gerber (8), foals infected with EHV-1 respond primarily with CMI; in contrast, older horses develop chiefly a humoral response. Other studies failed to show a clear CMI response to EHV-1 antigens in conventional and gnotobiotic foals (9, 90). CMI response of pregnant mares is suppressed in the last part of gestation, the ability of mounting a humoral response being unaffected (8, 10). The idea that immunosuppression in the pregnant mare might be related to fetal infection and abortion is tempting. The only example of CMI unresponsiveness in the horse is associated with combined immunodeficiency disease. However, a description of an immunodeficiency in a 17-month-old foal exposed to rhinopneumonitis virus during its fetal life gives some credit to the idea of a relation between immunodeficiency and EHV-1 infection.

FUNCTIONAL AND ULTRASTRUCTURAL EVALUATION OF NEUTROPHILS FROM FOALS AND LACTATING AND NON-LACTATING MARES

SUMMARY

Neutrophils from 4 pony foals, 3 lactating pony mares and 3 non-lactating mares were evaluated ultrastructurally and by *in vitro* function tests. Neutrophils from foals had significantly less random migration than neutrophils from mares; values in tests for iodination and *Staphylococcus aureus* ingestion were also lower with foal neutrophils but these differences did not reach statistical significance. Neutrophils from lactating mares had lower responses to iodination, antibody-dependent cell mediated cytotoxicity, and random migration tests than neutrophils from non-lactating mares. These differences were not statistically significant. Ultrastructurally, granule concentration did not differ significantly among groups. A slight decrease in primary granules and a corresponding increase in granules with a flocculent matrix suggests partial spontaneous neutrophil degranulation in foals and lactating mares.

INTRODUCTION

The low resistance to infection that occurs in early life of man and animals has been attributed to immaturity of specific immune defense mechanisms and to inadequate inflammatory responses, e.g., deficient phagocytosis (32, 91). Most animal research dealing with host defense mechanisms has focused on specific activities or products of the lymphoid system. Among non-specific mechanisms of immunity in neonates, the neutrophil plays a critical role although the manner by which dysfunctions lead to infection is largely unexplored.

The relation between neutrophil deficits and susceptibility to bacterial diseases is clear. Acquired deficits of neutrophils in immunosuppressive and antineoplastic therapy lead to overwhelming septic processes. Furthermore, rare congenital diseases characterized by neutrophil abnormalities, such as cyclic hematopoiesis and Chediak-Higashi syndrome, are associated with chronic bacterial infections (32, 92).

In vitro assays are commonly used in human medicine that measure various aspects of migration, phagocytosis, and bactericidal activity of neutrophils (35, 50, 93). Similar tests have been developed in domestic animals (17, 94, 95). In particular, chemotaxis (74), chemiluminescence (73), and antibody-dependent cell mediated cytotoxicity (ADCC) (75) have been measured in adult horses.

Neutrophils from newborn humans, tested in vitro, have deficient locomotion when compared to neutrophils from adults (96, 97, 98, 99).

This abnormality can contribute significantly to enhanced susceptibility of newborns to microbial invasion (98). Neutrophils from young animals have not been studied in that regard, except in rabbits where chemotaxis is also reduced (98).

In this study, we compared morphological and functional parameters between neutrophils from 2- to 4-month old foals to lactating and non-lactating mares. In order to determine the influence of eosinophils on function tests, assays were also run with granulocytes (neutrophils and eosinophils).

MATERIALS AND METHODS

Experimental design Neutrophils were evaluated ultrastructurally. Motility of neutrophils was measured by random migration on agarose. Phagocytosis was evaluated by measure of ^{125}I retained by phagocytized radiolabeled Staphylococcus aureus. Iodination of opsonized zymosan was used as a screening test of oxidative metabolism, which in turn is an indication of bactericidal activity of neutrophils (23). The potential role of neutrophils in cell mediated immunity was evaluated by ADCC.

Ten horses were divided in three groups: Group 1: Three lactating pony mares. Group 2: Four suckling, 2- to 4-month-old pony foals (3 were born from mares of group 1), and Group 3: Three non-lactating mares (2 Thoroughbreds and 1 pony). Animals were housed individually, except for lactating mares that remained with their foals. All animals were clinically normal during the experiment.

Blood (100 ml) was collected in sterile bottles containing 11 ml ACD (44 gm Trisodium citrate, 16 gm citric acid, 50 gm dextrose, 1000 ml triple distilled water). Erythrocytes were allowed to sediment for 45 minutes, and leukocyte-rich plasma was harvested and centrifuged at 1500 g for 5 minutes. Erythrocytes were lysed with hypotonic ammonium chloride in Tris buffer (70) and granulocytes were separated on Ficoll-Hypaque[a] (density 1.078) by centrifugation at 250 g for 20 minutes (70).

---

[a]Ficoll 400, Pharmacia Fine Chemicals, Piscataway, NJ: Hypaque Sodium, Winthrop Laboratories, New York, NY.

Granulocytes (neutrophils + eosinophils) were counted and resuspended to a final concentration of 5×10^7 per ml in Hanks' balanced salt solution without calcium and magnesium. This preparation was used in all assays using granulocytes. In assays using pure neutrophils, a second Ficoll-Hypaque gradient (density 1.125) was used (70). Centrifugation, cell counts, and resuspension were done as described for granulocytes.

The horses were all bled the same day, and all function tests were run in duplicate. The procedure was repeated 3 to 5 times. Smears of whole blood, granulocytes (neutrophils + eosinophils) and neutrophils were stained with May-Grünwald Giemsa for differential white blood cell counts and neutrophil-lymphocyte ratios.

Morphology Ten mls of blood were centrifuged at room temperature at 120 g for 10 minutes. Cold (4° C) glutaraldehyde (2.5%) was layered over the buffy coat layer. After 2 hours fixation, the buffy coat layer was removed, cut in 1 mm^3 pieces, placed in cacodylate buffer, and processed for electron microscopy (70). Six micrographs of individual neutrophils from each horse were taken at 13,000 x. Total and differential granule counts were done according to morphological criteria (70). Granule density per square micron of cytoplasm was determined for each cell and for each type of granule as follows:

$$\text{Granule density per } \mu m^2 = \frac{\text{Total granule number per cell}}{\text{Total cell area minus nucleus area (in } \mu m^2)}$$

Statistics Statistical analysis for each function test included mean ($\bar{x}$), variance (S^2), and standard error of the mean (SEM) determinations for each animal group. Comparisons between means (F tests) were

done on a daily basis. Granule numbers for each animal were determined for each neutrophil. Standard error of the mean values were generated from individual means. Percentage granules of each type were calculated for each animal group; total number of granules in each group was considered equal to 100%.

Random migration Migration of neutrophils was tested in 60 mm diameter petri plates containing 0.8% agarose in medium 199[b] with 10% horse serum (94). Neutrophils (5.0×10^5) were incubated for 12 hours at 37° C and then fixed with 2.5% glutaraldehyde, and stained with Wright's stain. Areas (mm^2) of neutrophil migration were determined using a semi-automatic digitizing tablet.[c] For each assay, the migration area was determined by difference between the total area covered by neutrophils minus the area occupied by the central well.

Staphylococcus aureus ingestion S. aureus ingestion was evaluated by a modification of a procedure previously reported for bovine neutrophils (94). S. aureus cells (labeled with ^{125}I) were added to bovine anti-staphylococcal antibodies and incubated with neutrophils (bacteria/neutrophil ratio: 60/1) for 45 minutes with constant agitation. The reaction was stopped by the addition of 0.5 unit of lysostaphin. Percent S. aureus ingestion was calculated as previously described (94).

Iodination Neutrophils (2.5×10^6) were mixed with 40 nmole NaI, 0.05 μCi ^{125}I, and 0.5 mg zymosan preopsonized with bovine serum in

---

[b]Grand Island Biological Co., Grand Island, NY.

[c]Bioquant II, R and M Biometrics, Nashville, TN.

a total volume of 0.5 ml Earle's balanced salt solution (94). The mixture was rotated (20 times per minute) for 20 minutes at 37° C; the reaction was terminated by adding cold 10% trichloracetic acid (TCA) at 4° C. Radioactivity was determined in a gamma counter and the amount of iodide converted to a TCA precipitable form was calculated. Appropriate controls were run as previously described (94).

Antibody dependent cell mediated cytotoxicity (ADCC) Bovine anti-chicken erythrocyte antibody was incubated with ^{51}Cr labeled chicken erythrocytes and 2.5 x 10^6 neutrophils for 120 minutes at 37° C (95). After incubation, tubes were centrifuged at 4° C, and 200 µl aliquots of supernatant were placed in a gamma counter for ^{51}Cr determination. Controls for each test included 100% ^{51}Cr release by addition of 1% Triton X100 to target cells and determination of background values by deletion of neutrophils and antibodies (95).

RESULTS

Differential white blood cell (WBC) counts of purified neutrophils after 2 Ficoll-Hypaque density gradient separations were 99% neutrophils and 1% eosinophils in the mares; 99.3% neutrophils, 0.5% eosinophils, and 0.2% lymphocytes in the foals. Neutrophil, lymphocyte, and total WBC counts in peripheral blood are given in Table 1. Neutrophil recovery after 2 Ficoll-Hypaque density gradients was about 10% total blood neutrophils.

Areas of random migration of neutrophils from foals were significantly smaller than areas of migration of neutrophils from lactating mares and non-lactating mares (Table 2). Values for iodination of opsonized zymosan and ingestion of S. aureus were lower with foal than with mare neutrophils. Values for ADCC were lowest with neutrophils from lactating mares (Table 2). Differences between horse group means were not statistically significant ($P>0.05$) for iodination, S. aureus ingestion, and ADCC (Table 2).

Morphologically, equine neutrophils from all groups were very similar. They were characterized by a round profile with thin cytoplasmic surface projections about 0.5 microns long. Cytoplasm matrices were dense, finely granular, and had scattered foci of glycogen particles. Nuclear lobes were round or kidney shaped, with an electron dense peripheral heterochromatin and electron lucent central euchromatin sometimes in contact with a nuclear pore. Four types of granules were seen. Primary granules were large, oblong, with a dense, sometimes

Table 1. Total WBC and neutrophil counts from peripheral blood in 3 horse groups

	Non-lactating mares	Lactating mares	Foals
Total WBC[a]	7141 ± 497 (3)[b]	7559 ± 1529 (3)	9781 ± 1775 (4)
Neutrophils[c]			
Segmented	53 ± 6.2 (3)	56.3 ± 7.2 (3)	28.5 ± 7.8 (4)
Bands	0	1	0
Lymphocytes[c]	41 ± 3.1 (3)	43 ± 7.5 (3)	70.7 ± 8.0 (4)
Neutrophils/ lymphocytes ratio	1.29	1.31	0.40

[a] In cells/mm^3.

[b] Mean ± standard error of the mean (number of horses).

[c] In percent.

Table 2. Results of function tests of horse neutrophils

	Non-lactating mares	Lactating mares	Foals
Random migr.[a]	17.49 ± 2.23 (6)[b]	14.79 ± 1.47 (6)	10.42 ± 0.49 (6)*
Staphylococcus aureus ingestion[c]	27.67 ± 3.49 (8)	27.72 ± 2.40 (9)	22.91 ± 3.45 (11)
Iodination[d]	22.69 ± 2.94 (11)	20.08 ± 1.73 (15)	18.39 ± 0.98 (22)
ADCC[e]	70.67 ± 5.37 (8)	54.56 ± 6.45 (12)	63.59 ± 3.92 (13)

[a] In square millimeters.

[b] Mean ± standard error of the mean (number of assays).

[c] In percent ingestion.

[d] In nMole NaI/10^7 neutrophils/20 minutes.

[e] In percent ^{51}Cr release.

* Statistically significant ($P<0.05$).

granular matrix. Secondary granules were elongated, electron dense, and frequently found in clusters. Granules with a flocculent matrix were seen in all cells and, occasionally, granules containing a crystalline structure in the matrix were also seen (Fig. 1). Concentration of total granules calculated in each group (Table 3) was higher in foals than in mares. Lactating mare and foal neutrophils had a higher percentage of flocculent granules and a lower percentage of primary granules than neutrophils from non-lactating mares (Table 4).

Granulocyte counts (neutrophils and eosinophils) after 1 Ficoll-Hypaque density gradient separation were 84.8% neutrophils, 15.0% eosinophils, 0.2% lymphocytes in the mares, 96.3% neutrophils, 3.5% eosinophils, 0.2% lymphocytes in the foals. Iodination of opsonized zymosan by foal granulocytes was significantly ($P<0.05$) lower than iodination by mare granulocytes (Table 5).

Percentage of ^{51}Cr release in the ADCC reaction was higher with foal granulocytes (neutrophils and eosinophils) than with mare granulocytes (Table 5). Ingestion of S. aureus by foal granulocytes was less than ingestion observed with mare granulocytes (Table 5). These differences were not significant ($P<0.05$).

Fig. 1. Horse neutrophil. Large ovoid primary granules (P), clusters of small, elongated, secondary granules (S), and granules with a flocculent matrix (F). Note the dense granular cytoplasm, devoid of granules in the center of the cell and under the cell membrane

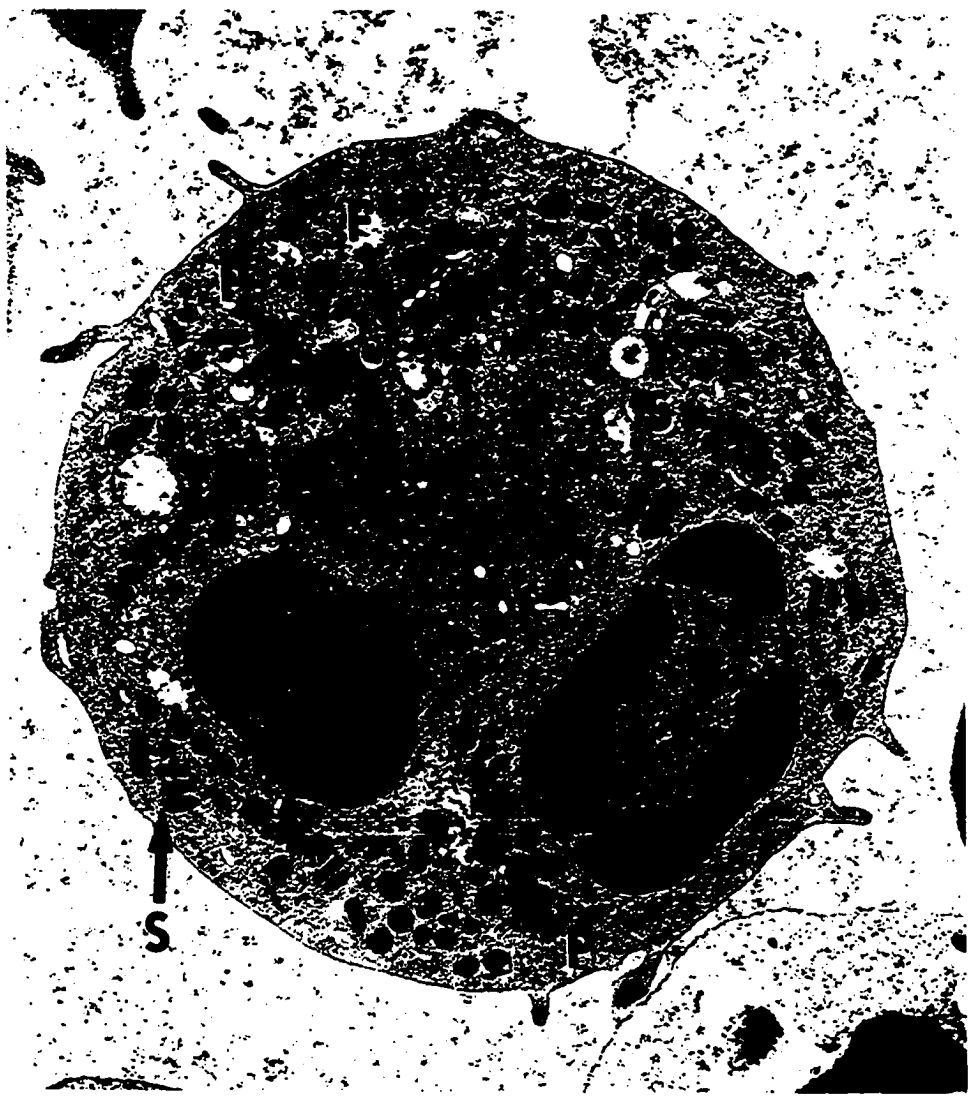

S

Table 3. Number of granules per square micron of neutrophil cytoplasm

Granule type	Non-lactating mares	Lactating mares	Foals
Primary	0.67 ± 0.03 (18)[a]	0.63 ± 0.02 (18)	0.68 ± 0.05 (24)
Secondary	4.97 ± 0.17 (18)	5.22 ± 0.09 (18)	5.76 ± 0.21 (24)
Flocculent matrix	0.69 ± 0.02 (18)	0.81 ± 0.01 (18)	0.86 ± 0.04 (24)
Crystalline matrix	0.01 ± 0.00 (18)	0.05 ± 0.00 (18)	0.03 ± 0.01 (24)
Total	6.37 ± 0.11 (18)	6.71 ± 0.12 (18)	7.35 ± 0.22 (24)

[a]Mean ± standard error of the mean (number of neutrophils).

Table 4. Percentage of different granule types in each horse group

Granule type	Non-lactating mares	Lactating mares	Foals
Primary	10.38 ± 0.68 (18)[a]	9.36 ± 0.17 (18)	9.12 ± 1.10 (24)
Secondary	78.64 ± 1.50 (18)	77.77 ± 0.09 (18)	79.19 ± 1.33 (24)
Flocculent matrix	10.77 ± 0.62 (18)	12.13 ± 0.20 (18)	11.58 ± 0.81 (24)
Crystalline	0.21 ± 0.07 (18)	0.75 ± 0.08 (18)	0.11 ± 0.03 (24)

[a]Mean ± standard error of the mean (number of neutrophils).

Table 5. Results of function tests of horse granulocytes

	Lactating mares	Foals
Staphylococcus aureus ingestion[a]	12.55 ± 8.15 (6)[b]	8.86 ± 2.79 (8)
Iodination[c]	72.52 ± 10.01 (6)	39.52 ± 6.45 (8)*
ADCC[d]	64.15 ± 10.68 (6)	69.55 ± 7.58 (8)

[a] In percent ingestion.

[b] Mean ± standard error of the mean (number of assays).

[c] In nMole NaI/10^7 granulocytes/20 minutes.

[d] In percent ^{51}Cr release.

* Statistically significant ($P<0.05$).

DISCUSSION

Our data, that foal neutrophils have impaired motility, suggest that neutrophil deficits may account for part of the sensitivity of foals to infections. Impaired neutrophil motility in human infants, is considered to significantly increase the susceptibility to infections (98). Reduced motility is related to a lack of membrane elasticity and impaired ability to redistribute cell membrane adhesion sites during migration (96, 100). Neutrophils from children with recurrent episodes of pneumonia have decreased chemotaxis, whereas, neutrophils from adults with pneumonia have increased chemotaxis, suggesting a primary neutrophil deficiency (101).

From this study, it appears that differences seen *in vitro* correspond to a primary neutrophil deficiency in normal foals. Neutrophil assays were done in absence of equine serum, precluding interference of serum factors with the results (93). The low percentage of morphologically immature forms (bands) in peripheral blood (Table 1) indicates that functional differences of foal neutrophils are not due to premature release from the bone marrow. The lower degree of *S. aureus* phagocytosis by foal neutrophils is consistent with neutrophil studies in humans, where phagocytosis of starch granules by neutrophils from neonates is lower than with neutrophils from adults (98).

The 19% decrease in iodination seen with foal neutrophils compared to non-lactating mares suggests a lower oxygen dependent microbicidal activity of foal neutrophils. Iodination of particles by neutrophils is

the result of a complex metabolic process including membrane oxidase activation, hydrogen peroxide production, and release of primary granules content (23, 51).

Flocculent granules are considered to be partially degranulated primary granules. Therefore, the lower percentage of primary granules and the presence of more flocculent granules in foals and lactating mares than in non-lactating mares (Table 5) suggests partial degranulation and lysosomal membrane instability. Partial degranulation in neutrophils from lactating mares and foals may account for the low iodination values observed in these two groups.

Breed differences are probably not a source of variation of neutrophil responses. Two mares in the non-lactating mare group were Thoroughbred, but no difference was observed between pony mare and Thoroughbreds.

Lactation was associated with a slight inhibitory effect on neutrophil functions. Although differences were not statistically significant, neutrophils from lactating mares were less responsive than neutrophils from non-lactating mares in all but one test. The most striking difference was seen with ADCC. Antibody-dependent cell mediated cytotoxicity is considered to be a mechanism of cell-mediated immunity against viruses (17). Decreased cell mediated immunity (8) and neutrophil dysfunction (102) occur in late pregnancy. It is possible that perturbations of the immune system associated with pregnancy still last during the lactation period. This aspect of immunity has not been adequately studied in animals.

A high peroxidase content of equine eosinophils is suggested by the greater iodination activity of a mixed granulocyte population relative to purified neutrophils. The presence of 15% more eosinophils resulted in increased iodination in mares by 73%. Cattle eosinophils have a 4- to 5-times higher iodination value than bovine neutrophils, due to a higher peroxidase content of the cell (95). In humans, eosinophils contain 2.6 times more peroxidase than neutrophils (103). These findings emphasize the need for separation procedures that give pure cell populations - in particular, removal of eosinophils is essential to an adequate evaluation of neutrophil antibacterial activity.

FUNCTIONAL AND ULTRASTRUCTURAL CHANGES IN NEUTROPHILS FROM MARES AND FOALS EXPERIMENTALLY INOCULATED WITH A RESPIRATORY STRAIN OF EQUINE HERPESVIRUS 1

SUMMARY

Neutrophils isolated from venous blood of adult and foal ponies inoculated with equine herpesvirus 1 were evaluated by *in vitro* function tests and by electron microscopy. Foals had fever and severe neutropenia 24 hours after inoculation; increased neutrophil random migration under agarose and decreased ADCC were significant at 24 hours but values had returned to normal by 72 hours. Mares had fever and leukopenia of less severity but increases in neutrophil migration and primary granule release persisted longer than in foals. Shorter migration increase, shorter and less severe decrease in primary granules, and decrease in ADCC seen with neutrophils from foals can relate to the high susceptibility of foals to EHV-1 infection.

INTRODUCTION

Equine herpesvirus 1 (EHV-1) or rhinopneumonitis virus, causes an acute catarrhal respiratory disease in foals. Severity of the disease is age related. Experimental and natural infection of foals during the first year of life can lead to pneumonitis, regional lymphadenitis, and transient leukopenia (9). Pneumonitis is often complicated by secondary bacterial infections, which can result in acute purulent bronchopneumonia. Hemolytic streptococci, Pasteurella hemolytica, and Escherichia coli are most often responsible for secondary pneumonia and death. Respiratory strains of EHV-1 are genetically and serologically distinct from abortigenic strains and have been associated with the respiratory disease of foals (6).

Neutrophils play a major role in recovery from cytolytic herpesvirus infections (16, 17). Although part of their effect relates to prevention of secondary bacterial infections, neutrophils also kill viruses by direct phagocytosis (15) and by antibody dependent cell mediated cytotoxicity (ADCC) (17, 66, 104, 105).

Neutrophils from normal foals, compared with adult horses, have a significantly reduced motility.[a] This reduced motility of neutrophils may be responsible for an increased susceptibility to respiratory infections, as reported in human infants (98). In addition, the ability of neutrophils to limit virus spread and control secondary bacterial

---

[a]F. L. Coignoul et al., Am. J. Vet. Res., in press.

MATERIALS AND METHODS

Experimental design Three groups of horses were used in this experiment. A first group (inoculated mares) was composed of 3 lactating pony mares that were inoculated by intranasal (10 ml) and intravenous (6 ml) routes with a respiratory strain of EHV-1 (6.3×10^5 plaque forming units/ml). Each mare was inoculated intranasally with 5 ml virus suspension from a syringe without needle introduced in each nostril. The head of the mare was held up during the inoculation procedure and for one additional minute after inoculation. The same mare was then inoculated intravenously in the jugular vein with a virus suspension of the same titer. Another group (foals) was composed of 4 2- to 4-month old pony foals. Each foal was inoculated with the virus as described for mares of group 1. The third group, (non-inoculated mares) was composed of 2 Thoroughbred mares and 1 pony mare that were not inoculated. Body temperatures were taken twice daily. Total white blood cell (WBC) counts were determined daily. One hundred ml of blood from each animal was collected in sterile bottles containing 11 ml acid-citrate dextrose 1 day before inoculation and on days 1, 2, 3, 5, and 6 post inoculation (94). Neutrophils were separated on 2 successive Ficoll-Hypaque[b] density gradients (70) and used in all function tests. Each test was run in duplicate.

---

[b]Ficoll 400, Pharmacia Fine Chemicals, Piscataway, NJ; Hypaque Sodium, Winthrop Laboratories, New York, NY.

Neutrophils from 2 inoculated mares and 2 foals taken 1 day before inoculation and on days 1, 3, and 5 after inoculation, were examined with an electron microscope.

Virus Strain 76-10356 of EHV-1 subtype 2 was obtained from Dr. J. Pearson (National Veterinary Services Laboratories, Ames, Iowa). Virus was propagated in sub-confluent equine dermis (ED) cell cultures in plastic flasks. After 48 hours, infected cell cultures were frozen, thawed, and centrifuged at 800 g for 5 minutes. Supernatant was stored at -60° C and used for horse inoculations.

Cell cultures Equine dermis cells and primary equine embryonic kidney (EEK) cells, at 5th or 6th culture passage, were used. For both cell types, growth medium was Eagle's MEM supplemented with 15% fetal calf serum (FCS), 10% L-glutamine, and 75 µg/ml gentamycin.[c] Maintenance medium was identical to growth medium except the FCS was at 10%.

Antiviral activity Antiviral and cellular immunity functions of neutrophils were evaluated by the ADCC test. Bovine anti-chicken erythrocyte antibody was incubated with ^{51}Cr labeled chicken erythrocytes and 2.5×10^6 neutrophils for 120 minutes at 37° C (95). After incubation, tubes were centrifuged at 4° C and 200 µl aliquots of supernatant were placed in a gamma counter for ^{51}Cr determination. Controls for each test included 100% ^{51}Cr release by addition of 1% Triton X100 to target cells and determination of background values by omitting neutrophils and antibodies from the test (95).

---

[c]Gentocin® Schering, Kenilworth, NJ.

Motility Motility of neutrophils was evaluated by measuring random migration under agarose. Tests were done in 60 mm diameter petri plates containing 0.8% agarose in medium 199[d] with 10% horse serum (94). Neutrophils (5.0×10^5 per assay) were incubated for 12 hours at 37° C, then fixed with 2.5% glutaraldehyde, and stained with Wright's stain. For each assay, the migration area (mm^2) was determined by difference between the total area covered by neutrophils minus the area occupied by the central well. Areas were determined using a semi-automatic digitizing tablet.[e]

Phagocytosis Phagocytic activity of neutrophils was evaluated by a modified procedure using iododeoxyuridine (^{125}I) labeled Staphylococcus aureus (94). Labeled S. aureus were added to bovine anti-staphylococcal antibodies and incubated with neutrophils (bacteria/neutrophil ratio 60/1) for 45 minutes with constant agitation. The reaction was stopped by addition of 0.5 unit of lysostaphin. Percent S. aureus ingestion was calculated as described (94).

Iodination assays Iodination of opsonized zymosan was used as a screening test of oxidative metabolism and related bactericidal activity of neutrophils (95). Neutrophils (2.5×10^6 cells) were mixed with 40 nmole NaI, 0.05 μCi ^{125}I, and 0.5 mg zymosan preopsonized with bovine serum in a total volume of 0.5 ml Earle's balanced salt solution (94). The mixture was rotated (20 times per minute) for 20 minutes at

---

[d]Grand Island Biological Co., Grand Island, NY.

[e]Bioquant II, R and M Biometrics, Nashville, TN.

37° C, the reaction was terminated by adding cold (4° C) 10% trichloracetic acid (TCA). Radioactivity was determined in a gamma counter and the amount of iodide converted to a TCA precipitable form was calculated. Controls were run as previously described (94).

Morphology The morphology of neutrophils was evaluated with an electron microscope. For each examination, 10 ml of blood was centrifuged at room temperature, at 120 g for 10 minutes and cold (4° C) glutaraldehyde (2.5%) was layered over the buffy coat. Fixed cells were processed for transmission electron microscopic examination (70). Six micrographs of individual neutrophils from each horse were taken at 11,500 x. Granule concentration of each granule type per square micron of cytoplasm was determined for each cell as follows:

$$\text{Granule concentration per } \mu m^2 = \frac{\text{Total granule number per cell}}{\text{Total cell area minus nucleus area (in } \mu m^2)}$$

Areas were determined using the semi-automatic digitizing tablet.

Statistical analysis The mean ($\bar{x}$), variance (S^2), and standard error of the mean (SEM) were determined for each function test. Comparisons of means (F tests) were done among horse groups on a daily basis. Percentages of granule types were calculated for each horse group. Comparisons among groups were done between daily means (F tests) before and after inoculation.

RESULTS

After exposure to EHV-1, the inoculated mares and foals had leukopenia and fever within 24 hours. Fever lasted for 24 to 36 hours and WBC counts returned to normal in 24 to 48 hours. Non-inoculated mares had normal temperatures and WBC counts during the entire observation period.

Antibody dependent cell mediated cytotoxicity was not significantly different among the 3 groups before inoculation (Fig. 1). With neutrophils from foals, percentage ADCC was significantly ($P<0.01$) less than with neutrophils from non-inoculated mares on day 1 post-inoculation. Three days after inoculation, ADCC by neutrophils from foals was similar to ADCC values before inoculation. On day 6, percentage ADCC with neutrophils from foals was greater than ADCC with neutrophils from non-inoculated mares. Percentage ADCC with neutrophils from inoculated mares was not, either before or after inoculation, significantly different from non-inoculated mares.

Prior to inoculation, areas of random migration were less with neutrophils from foals ($P<0.01$) and inoculated mares ($P<0.05$) than with neutrophils from non-inoculated mares (Table 1). Increased migration was seen 48 hours after inoculation in all foals. Three days after inoculation, neutrophil migration in 3 of the 4 foals had returned to preinoculation levels. Areas of neutrophil migration in inoculated mares was slightly greater than areas of migration of neutrophils from non-inoculated mares up to 5 days after inoculation.

Fig. 1. Antibody-dependent cell mediated cytotoxicity (ADCC) by neutrophils from mares and foals inoculated with EHV-1 (infected mares and infected foals) and from non-inoculated mares (control horses). Figures are averaged on 100% ADCC by non-inoculated mares on a daily basis. Day 0: preinoculation - Vertical bar = 2 x standard error of the mean

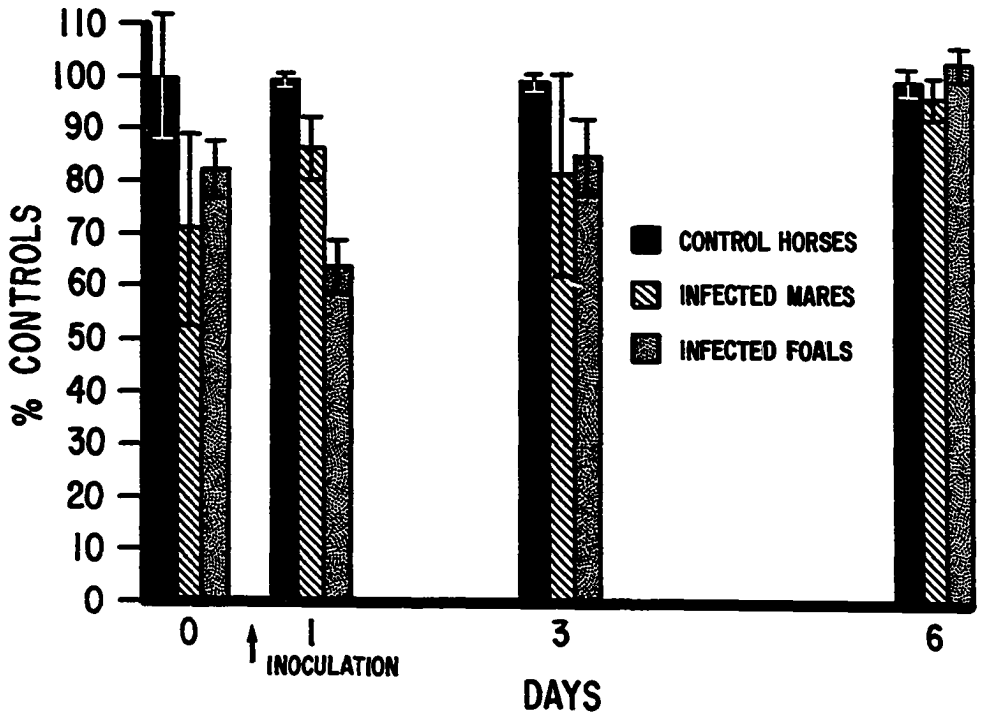

110
100
90
80
70
60
50
40
30
20
10
0
% CONTROLS
0
1
3
6
INOCULATION
DAYS
CONTROL HORSES
INFECTED MARES
INFECTED FOALS

Table 1. Horse neutrophils. Areas of random migration (in mm^2) under agarose. Principal foals (Foals) and mares (Mares) inoculated with a respiratory strain of EHV-1 and non-inoculated control mares (Controls)

Days	Foals	Mares	Controls
0[a]	9.65 ± 0.65 (4)[b]**	16.53 ± 1.01 (3)*	22.35 ± 0.04 (2)
1	-	-	-
2	20.58 ± 0.09 (4)	20.43 ± 0.07 (3)	20.53 ± 0.10 (3)
3	13.90 ± 2.74 (4)	19.76 ± 1.37 (3)	20.20 ± 0.07 (2)
4	-	-	-
5	14.40 ± 2.00 (4)	21.53 ± 0.28 (3)	19.10 ± 2.49 (3)

[a]Preinoculation values.

[b]Mean ± standard error of the mean (number of horses).

**Statistically significant ($P<0.01$).

* Statistically significant ($P<0.05$).

Phagocytosis of S. aureus was less by neutrophils from foals than from either inoculated or non-inoculated mares before inoculation (Table 2). Percentage phagocytosis by neutrophils from foals was higher than phagocytosis by neutrophils from non-inoculated mares on days 1 and 2 after inoculation. Phagocytosis of S. aureus by neutrophils from inoculated mares was greater than non-inoculated mares on days 1, 2, and 5, and less on day 3. None of the differences in S. aureus ingestion were significant.

Iodination of opsonized zymosan by neutrophils was similar in all groups before inoculation (Table 3). After inoculation, iodination by neutrophils from foals was less than iodination by neutrophils from non-inoculated mares on day 1 and greater on days 2 and 3. Iodination by neutrophils from inoculated mares was greater than from non-inoculated mares on days 2, 5, and 6 after inoculation. None of the iodination differences were significant.

Neutrophils from foals and mares had the characteristic morphology of unstimulated neutrophils before and after inoculation. Cells were round with regular contours and had irregular nuclear lobes in excentric position. The cytoplasm was dense, finely granular, and contained diffuse glycogen granules. Phagosomes and lipid vacuoles were rarely present. One to 3 mitochondria were seen in most cell sections. Primary granules, secondary granules, and flocculent matrix granules were present in all cells examined (Fig. 2). Before inoculation, granule concentration was not significantly different between mares and foals (Tables 4 and 5). Neutrophils from inoculated mares had a signif-

Table 2. Staphylococcus aureus ingestion (in percent phagocytosis) by neutrophils from foals (Foals) and mares (Mares) inoculated with a respiratory strain of EHV-1 and from non-inoculated control mares (Controls). None of the differences are statistically significant ($P<0.05$)

Days	Foals	Mares	Controls
0[a]	22.91 ± 3.45 (4)[b]	27.72 ± 2.40 (3)	27.67 ± 3.49 (3)
1	26.83 ± 5.08 (3)	32.23 ± 4.21 (3)	25.07 ± 4.19 (3)
2	22.88 ± 1.64 (4)	29.30 ± 1.26 (3)	22.10 ± 4.80 (3)
3	14.83 ± 3.06 (4)	13.17 ± 4.68 (3)	29.05 ± 1.75 (2)
4	-	-	-
5	-	18.93 ± 2.17 (3)	20.60 ± 2.60 (2)
6	23.85 ± 4.70 (4)	31.70 ± 4.30 (2)	31.63 ± 1.86 (3)

[a]Preinoculation values.

[b]Mean ± standard error of the mean (number of horses).

Table 3. Iodination of opsonized zymosan (in nm NaI/10^7 neutrophils/hour) by neutrophils from foals (Foals) and mares (Mares) inoculated with a respiratory strain of EHV-1 and from non-inoculated control mares (Controls). None of the differences are statistically significant ($P<0.05$)

Days	Foals	Mares	Controls
0[a]	18.39 ± 0.98 (4)[b]	20.08 ± 1.73 (3)	22.69 ± 2.94 (3)
1	12.98 ± 1.17 (4)	13.36 ± 1.52 (3)	15.53 ± 3.59 (3)
2	23.08 ± 1.27 (4)	22.80 ± 0.62 (3)	15.47 ± 2.26 (3)
3	20.03 ± 2.74 (4)	11.76 ± 2.98 (3)	15.40 ± 6.40 (2)
4	-	-	-
5	9.12 ± 2.06 (4)	19.93 ± 1.40 (3)	12.36 ± 2.09 (3)
6	18.22 ± 4.47 (4)	19.93 ± 2.43 (3)	18.86 ± 2.44 (3)

[a]Preinoculation values.

[b]Mean ± standard error of the mean (number of horses).

Fig. 2. Neutrophil from a mare of the inoculated group before EHV-1 inoculation. Note large ovoid primary granules (P), clusters of small dense secondary granules (S), empty and flocculent matrix granules (F). Large membrane bound spaces are mitochondria (*)

Table 4. Granule concentration (in granule number per sq. micron of cytoplasm) in neutrophils from 2 mares inoculated with a respiratory strain of EHV-1

Granule	Day 0[a]	Day 1	Day 3	Day 5
Primary	0.61 ± 0.04[b]	0.27 ± 0.04*	0.44 ± 0.08	0.29 ± 0.02*
Secondary	5.18 ± 0.26	4.69 ± 0.79	6.64 ± 0.54	5.73 ± 0.14
Flocculent	0.83 ± 0.04	0.20 ± 0.04**	0.45 ± 0.04*	0.47 ± 0.04*
Crystalline	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.00	0.02 ± 0
TOTAL	6.66 ± 0.35	5.19 ± 0.28	7.58 ± 0.66	6.50 ± 0.17

[a]Day 0: Preinoculation.

[b]Mean ± standard error of the mean of 12 neutrophils.

*Statistically significant ($P<0.05$).

**Statistically significant ($P<0.01$).

Table 5. Granule concentration (in granule number per sq. micron of cytoplasm) in neutrophils from 2 foals inoculated with a respiratory strain of EHV-1. None of the differences are statistically significant

Granule	Day 0[a]	Day 1	Day 3	Day 5
Primary	0.60 ± 0.15[b]	0.45 ± 0.05	0.40 ± 0.02	0.39 ± 0.04
Secondary	5.43 ± 0.58	6.21 ± 0.59	4.62 ± 0.30	5.23 ± 0.47
Flocculent	0.76 ± 0.09	0.28 ± 0.09	0.60 ± 0.05	0.68 ± 0.18
Crystalline	0.01 ± 0.00	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.01
TOTAL	6.85 ± 0.45	6.87 ± 0.62	6.50 ± 0.33	6.31 ± 0.68

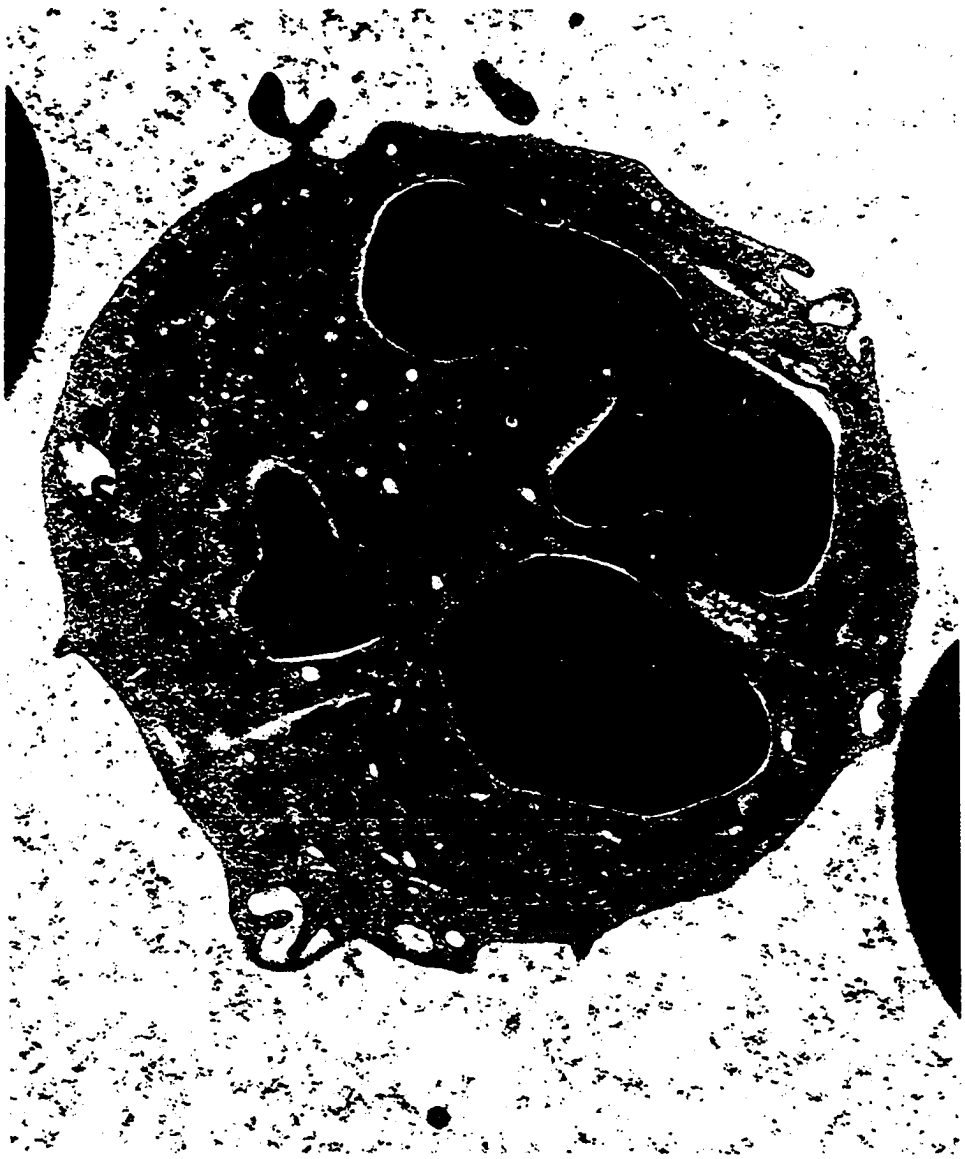
[a]Day 0: Preinoculation.

[b]Mean ± standard error of the mean of 12 neutrophils.

icant ($P<0.05$) decrease in primary granule concentration on days 1 and 5 after inoculation (Table 4). In some neutrophils from these mares, there was no evidence of primary granules (Fig. 3). A significant decrease in flocculent matrix granule concentration was also seen in neutrophils from these mares on day 1 ($P<0.01$) and on days 3 and 5 ($P<0.05$) after inoculation (Table 4).

Neutrophils from foals had a decrease in primary granule concentration on days 1, 3, and 5 after inoculation, and a marked decrease in flocculent matrix granule concentration 1 day after inoculation (Table 5). None of these differences were statistically significant.

Fig. 3. Neutrophil from a mare, 24 hours after EHV-1 experimental inoculation. Cytoplasm contains secondary granules (S) and no primary granule. Note the rarity of granules with a flocculent matrix (F)



DISCUSSION

In foals inoculated with EHV-1 subtype 2, there was a low neutrophil response characterized by transient increase in migration, significant decrease in ADCC, and mild decrease in granule concentration. This low neutrophil response may increase susceptibility to infectious agents.

Migratory stimulation of neutrophils, seen only for 48 hours after EHV-1 subtype 2 inoculation in 3 of the 4 pony foals, contrasted with the persistent increased migration seen with neutrophils from inoculated mares. This difference in duration can be due to the higher turnover rate and shorter intravascular lifespan of granulocytes in young animals (106). This observation and previous description of significantly less migration of neutrophils from normal, non-inoculated foals as compared to those from mares,[a] suggests possible inefficiency of neutrophils to marginate and migrate through blood vessel walls and toward areas of inflammation in foals exposed to infectious agents.

The significant decrease in ADCC, seen only with neutrophils from inoculated foals, could partly explain the severity of EHV-1 infection in foals. ADCC is an *in vitro* phenomenon of cellular immunity (16). It is believed to have an *in vivo* counterpart, involved in destruction of virus infected cells (16, 17). Impaired or delayed killing of infected cells, even of short duration, can give the virus a decisive advantage enabling infection of the host to take place.

---

[a] F. L. Coignoul *et al.*, Am. J. Vet. Res., in press.

Significant decrease in concentration of primary granules and flocculent matrix granules were seen in inoculated mares, indicating selective degranulation of primary granules. Decrease in granule concentration was seen only with these 2 granule types. Flocculent matrix granules of neutrophils formed a heterogeneous population, they had the size and shape of primary granules but contained various amounts of flocculent electron dense material. Flocculent matrix granules, abundant in non-stimulated neutrophils, have been previously considered to be altered primary granules (70). Therefore, a decrease in both granule types is indicative of a selective loss of primary granules. In normal phagocytosis, secondary granule degranulation precedes degranulation of primary granules. However, there is evidence that degranulation is a separate event for primary and secondary granules and that specific degranulation of one granule type can occur (107).

Alterations of neutrophil functions were not due to opsonins or mediators of inflammation from the blood. Testing neutrophils in vitro in the presence or absence of serum can distinguish between influences of serum factors and direct alterations of the cells (93). In this study, function tests were done in the absence of horse serum, indicating that EHV-1 affects directly the circulating neutrophil population.

Migration and degranulation depend extensively on the cytoskeleton, primarily microfilaments (108). As both of these functions were only transiently increased in neutrophils from foals and more permanently in neutrophils from mares, it is possible that EHV-1 infection triggered

neutrophil primary granule enzyme release, increased cell motility, and phagocytosis, possibly by interaction with the cytoskeleton.

Only mild clinical signs were seen in infected foals, typical of EHV-1 subtype 2. Subtype 1 is the abortigenic virus, and can be recovered from aborted fetuses, buffy coat, and feces (6). Subtype 2 growth in culture is less rapid, and is restricted to cells of equine and hamster origin (109, 110). EHV-1 subtype 2 has only been associated with respiratory disease (6). The strain used in our study was identified as a respiratory strain by restriction endonucleases DNA patterns (111). Influence of virus subtypes on defense mechanisms of the horse should be evaluated, particularly in view of developing an efficacious vaccine, safe for foals and for pregnant mares.

Differences in morphology and random migration seen before inoculation with neutrophils from the inoculated mares compared to non-inoculated mares is probably due to lactation.[a]

---

[a]F. L. Coignoul et al., Am. J. Vet. Res., in press.

PATHOGENICITY OF EQUINE HERPESVIRUS 1 SUBTYPE 2 FOR FOALS AND ADULT PONY MARES

SUMMARY

Three pony mares and 4 pony foals were inoculated with a subtype 2 strain of equine herpesvirus 1. Foals had periods of fever 12 hours and 3-1/2 days after inoculation, and leukopenia involving both neutrophils and lymphocytes, followed by leukocytosis. Inoculated mares had a transient leukopenia at 24 hours after inoculation; fever and leukopenia were less severe in mares than in foals. A fourfold increase in circulating virus neutralizing antibody was seen in 2 of 3 inoculated mares but none of the foals after inoculation. Attempts to recover virus from the blood were unsuccessful.

Equine herpesvirus 1 subtype 2 is a mild pathogen for ponies and infection may result in inapparent clinical disease. Poor serologic response and absence of demonstrable virus in the blood were consistent with the mild clinical disease.

INTRODUCTION

Two distinct subtypes of equine herpesvirus (1 EHV-1, equine rhinopneumonitis virus) are associated with various clinical diseases in horses. Subtype 1 (abortigenic virus) causes abortion (14), respiratory infections in foals (2, 3), and neurologic disease (4, 112). Virus strains of subtype 1 replicate in respiratory tract and intestinal epithelium. Virus can be recovered from leukocytes and feces of infected horses and from visceral organs of aborted fetuses (6). Strains of EHV-1 subtype 1 that replicate in endothelial cells of the central nervous system blood vessels are responsible for the neurologic disease (6, 7). Subtype 2 (respiratory virus) is genetically (111, 113) and serologically (110, 114) different from subtype 1. It grows less rapidly in cell cultures and only in cells of equine and hamster origin (109). Subtype 2 is associated with respiratory disease in foals only (6).

Studies of EHV-1 infection in foals have been conducted with EHV-1 subtype 1 (8, 11, 14, 115), with virus strains of unspecified subtype (3, 116), and with vaccinal strains that have lost the characteristic DNA pattern of EHV-1 subtype 2 (7, 114).

This study was undertaken to evaluate the clinical, serological and hematological responses of ponies to EHV-1 subtype 2 and to compare the response of foals and adult ponies to the virus.

MATERIALS AND METHODS

Experimental design Three groups of horses were used in this study: Group I, 3 pony mares (M7, M8, and M9); Group II, 4 suckling, 2- to 4-month old, pony foals (F6, F7, F8, and F9). Foals F7, F8, and F9 were born from mares of group I; and Group III, 2 Thoroughbred mares (C1 and C2) and 1 pony mare (C3), were non-inoculated controls. Horses of groups I and II were inoculated with EHV-1 subtype 2 by intranasal (10 ml) and intravenous (6 ml) routes (6.3×10^5 plaque forming units/ml). Each horse of Groups II and III was inoculated intranasally with 5 ml virus suspension from a syringe without needle introduced in each nostril. The head of the horse was held up during the inoculation procedure and for one additional minute after inoculation. Each horse was also inoculated intravenously in the jugular vein with a virus suspension of the same titer. Animals were housed individually, except for lactating mares (group I) that remained with their foals. Body temperatures were taken twice daily. Blood was collected one day before inoculation and days 1, 2, 3, 5, and 6 after inoculation for total and differential white blood cell (WBC) counts, hematocrits, virus neutralization (VN) tests, and virus isolations. Blood was collected at 20 and 43 days after inoculation for VN tests and virus isolations.

Virus Strain 76-10356 was obtained from Dr. J. E. Pearson.[a] Virus was originally isolated from the nasal tract of a horse in Iowa

---

[a]National Veterinary Services Laboratories, Ames, IA.

and was classified as a subtype 2 of EHV-1 by restriction endonuclease DNA patterns (111). Virus was propagated on sub-confluent cultures of equine dermis (ED) cells.[b] After 48 hours, infected cultures with evidence of cytopathogenic effect (CPE) were frozen, thawed, and pelleted by centrifugation at 800 g for 5 minutes. Supernatant fluid was stored at -60° C and used for inoculations.

Cell cultures Primary embryonic equine kidney (EEK) cells at 5th or 6th culture passage and ED cells were used for virus multiplication. For both cell types, growth medium was Eagle's MEM supplemented with 15% fetal calf serum (FCS), 10% L-glutamine, and 75 µg/ml gentamycin.[c] Maintenance medium was identical to growth medium, except the FCS was at 10%. Cell cultures were incubated at 37° C.

Hematology Ten ml citrated blood was used for hematocrit determinations and WBC counts. Total WBC counts were done with an electronic cell counter[d] and differential WBC counts were done on blood smears stained with May-Grünwald Giemsa stain.

VN tests Assays were done by a modified procedure using ED cells grown in 96 well flat bottom microtiter plates (90). Serum dilutions were done in Eagle's MEM and virus was added to the test. Challenge virus was EHV-1 subtype 1 obtained from J. E. Pearson.[a] After 3 days of incubation at 37° C, inhibition of CPE was assessed by

---

[b]American Type Culture Collection, Rockville, MD.

[c]Gentocin® Schering, Kenilworth, NJ.

[d]Coulter Counter Mod. B, Coulter Electronics, Hialeah, FL.

microscopy on unstained plates. Titers were expressed as the reciprocal of the test serum dilutions that gave 100% inhibition of CPE. A standard immune serum was incorporated in all tests.

Virus isolation Ten ml of blood were allowed to clot at 4° C and after serum was removed, blood clots were stored at -60° C. Frozen clots were ground in a TenBroek grinder with 8 ml cold (4° C) Eagle's MEM containing 75 µg/ml gentamycin. After centrifugation (1,300 g, 20 minutes, 4° C), supernatant fluid was frozen and stored (-60° C). Leighton tubes containing a confluent layer of EEK cells were inoculated in duplicate with 1 ml each of the thawed supernatant fluid. Cell cultures were examined for 1 week for evidence of CPE. A second passage, made by transfer of 0.1 ml freeze-thawed culture onto a fresh cell monolayer, was incubated for one week and examined for CPE.

Statistics Neutrophil-lymphocyte (N/L) ratio daily means were compared within each horse group for significant differences (F tests). Body temperature means and standard deviations were calculated daily in each horse group.

RESULTS

All challenged ponies developed fever after inoculation. Foals had a biphasic fever response. The first fever period began 12 hours after inoculation and lasted for 36 hours. The second fever period began 3-1/2 days after inoculation and was transient (Fig. 1). Inoculated mares had one fever peak that began in 2 mares at 12 hours after inoculation and lasted for 12 hours (Fig. 2). The third mare had fever that began at 24 hours after inoculation and lasted for 24 hours. Non-inoculated mares had normal (37.5 to 38.5^{o} C) temperatures during the entire observation period (Fig. 3).

All foals and 2 of 3 inoculated mares had a decrease in WBC's by 24 hours after inoculation (Fig. 4 and 5). One foal (F6) had leukocytosis before inoculation and relative leukopenia was seen between day 1 and day 5 after inoculation in this animal. In foals, total WBC counts returned to normal (6-12,000 WBC/µl) by 3 days after inoculation and by day 6, total WBC counts were 15 to 33% higher than before inoculation.

One inoculated mare (M7) had leukopenia that persisted during the entire observation period and was accentuated after inoculation. All WBC counts remained within normal limits in non-inoculated horses (Fig. 6).

In foals, N/L ratio was smaller than 1, except on day 3 after inoculation where it was significantly ($P<0.01$) increased (Table 1). In mares, the N/L ratio was greater than 1 except on the 5th and 6th days

Fig. 1. Average temperature in 4 pony foals experimentally inoculated with equine herpesvirus 1 subtype 2. Vertical bar = 2 X standard deviation

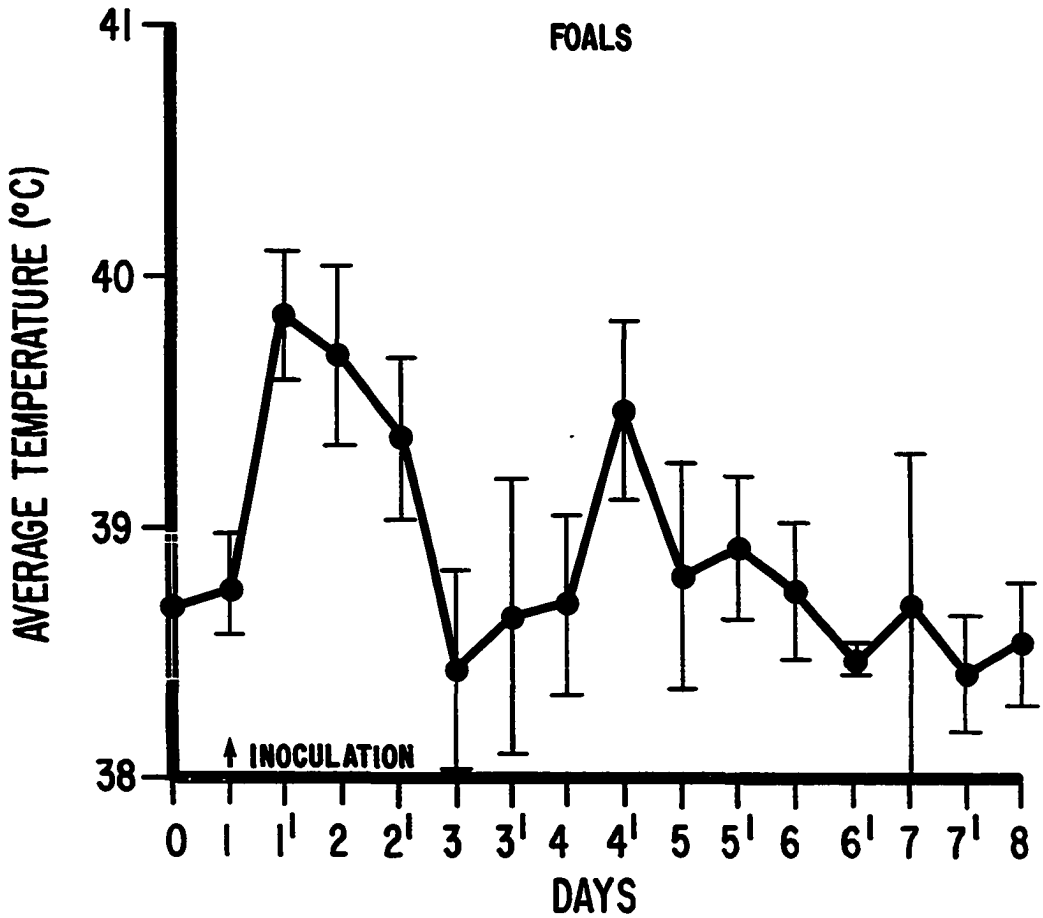

FOALS
AVERAGE TEMPERATURE (°C)
41
40
39
38
INOCULATION
0 1 1¹ 2 2¹ 3 3¹ 4 4¹ 5 5¹ 6 6¹ 7 7¹ 8
DAYS

Fig. 2. Average temperature in 3 pony mares experimentally inoculated with equine herpesvirus 1 subtype 2. Vertical bar = 2 X standard deviation

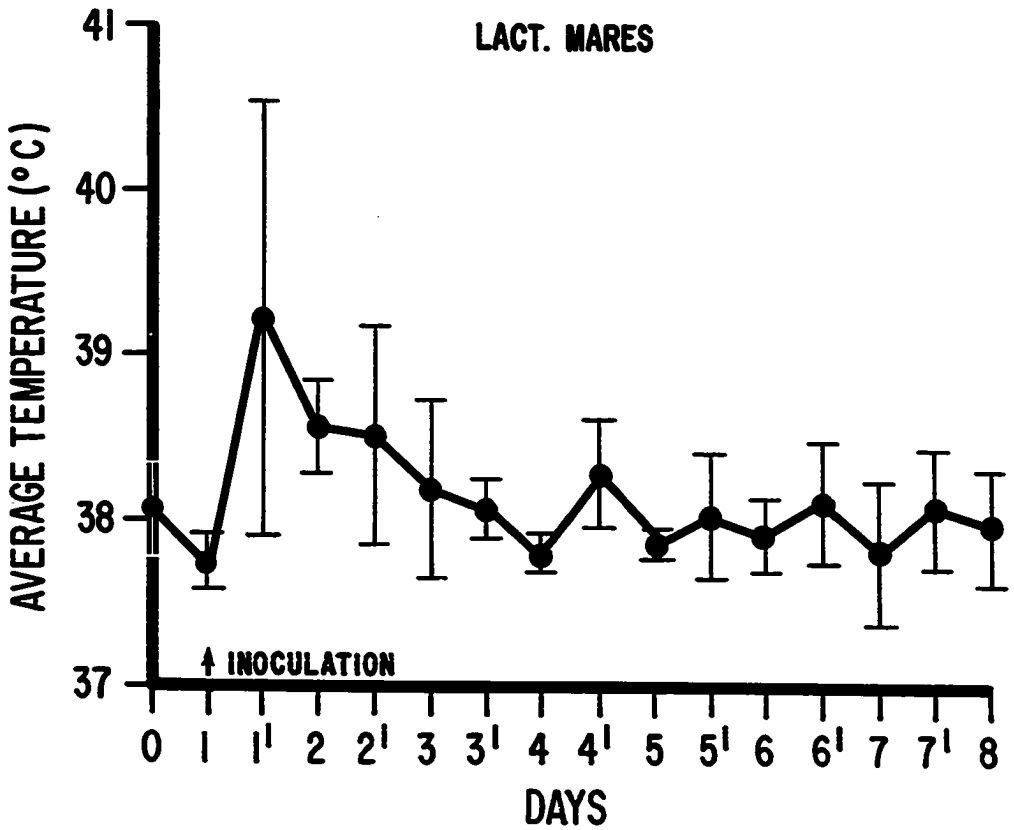

LACT. MARES
AVERAGE TEMPERATURE (°C)
41
40
39
38
37
INOCULATION
0 1 1' 2 2' 3 3' 4 4' 5 5' 6 6' 7 7' 8
DAYS

Fig. 3. Average temperature in 3 non-inoculated mares.

Vertical bar = 2 X standard deviation

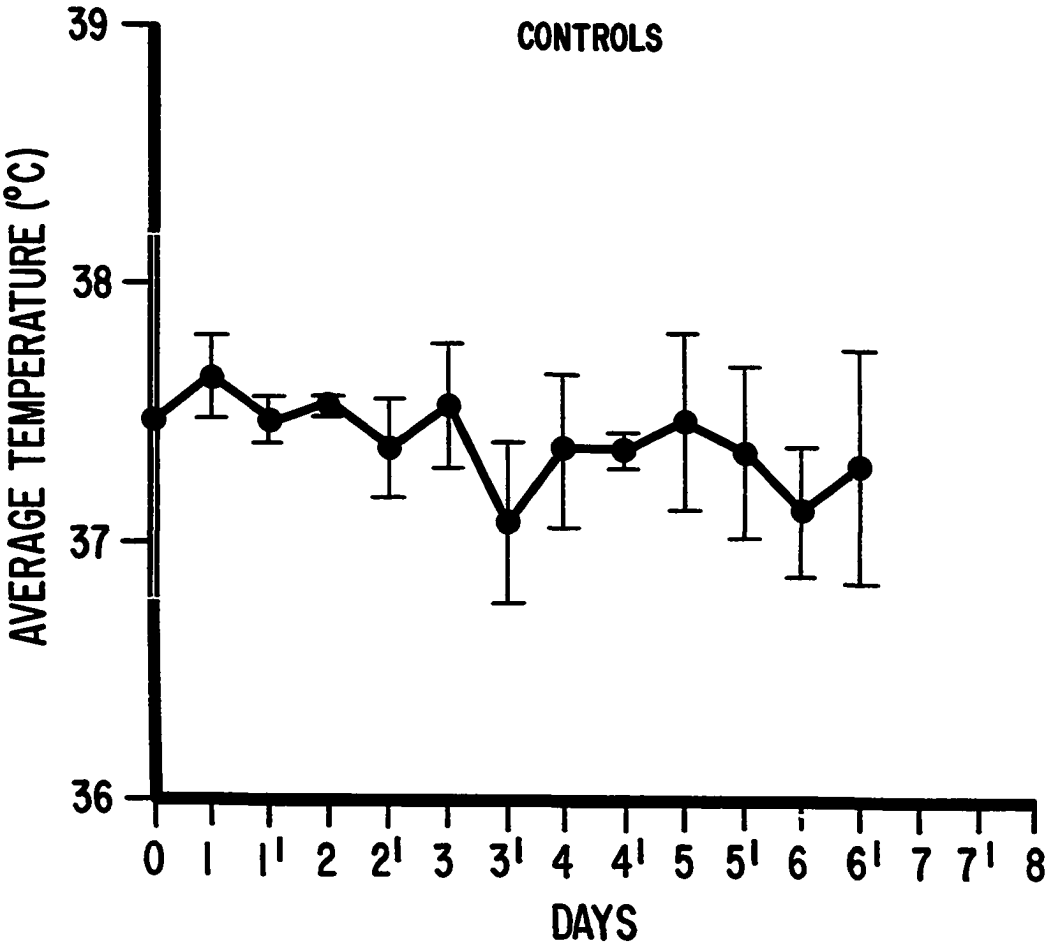

CONTROLS
AVERAGE TEMPERATURE (°C)
39
38
37
36
0 1 1¹ 2 2¹ 3 3¹ 4 4¹ 5 5¹ 6 6¹ 7 7¹ 8
DAYS

Fig. 4. Total white blood cell counts in 4 pony foals (F6, F7, F8, F9) experimentally inoculated with equine herpesvirus 1 subtype 2

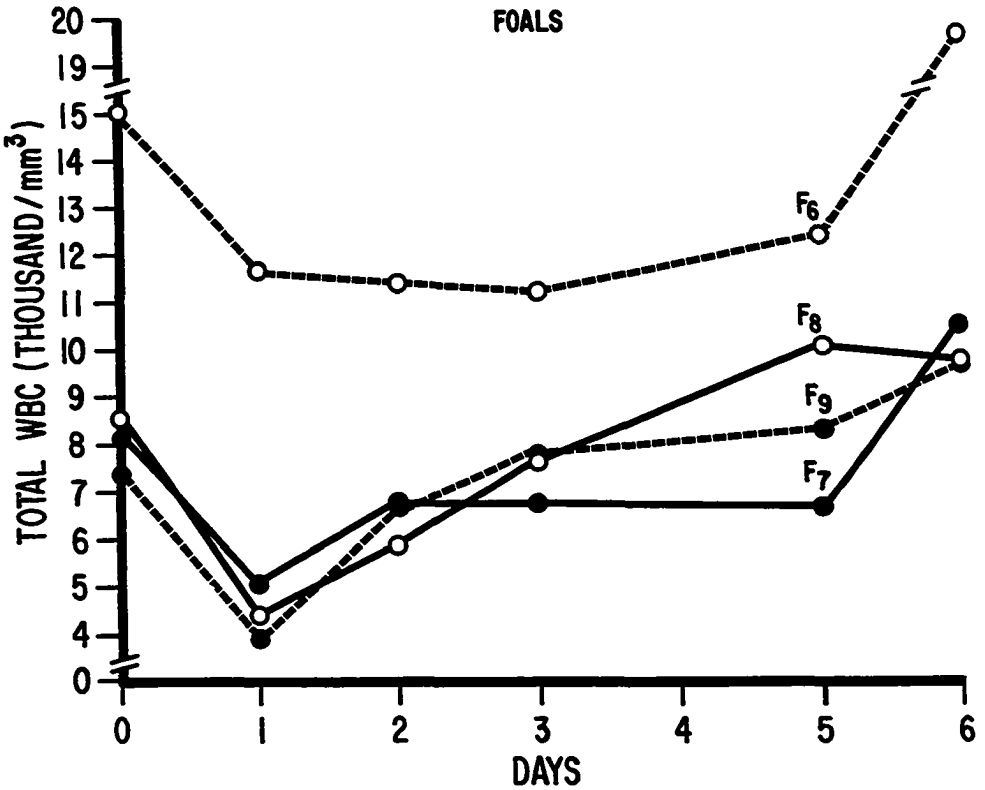

FOALS
TOTAL WBC (THOUSAND/mm³)
20
19
15
14
13
12
11
10
9
8
7
6
5
4
0
0
1
2
3
4
5
6
DAYS
F6
F8
F9
F7

Fig. 5. Total white blood cell counts in 3 pony mares (M7, M8, M9) experimentally inoculated with equine herpesvirus 1 subtype 2

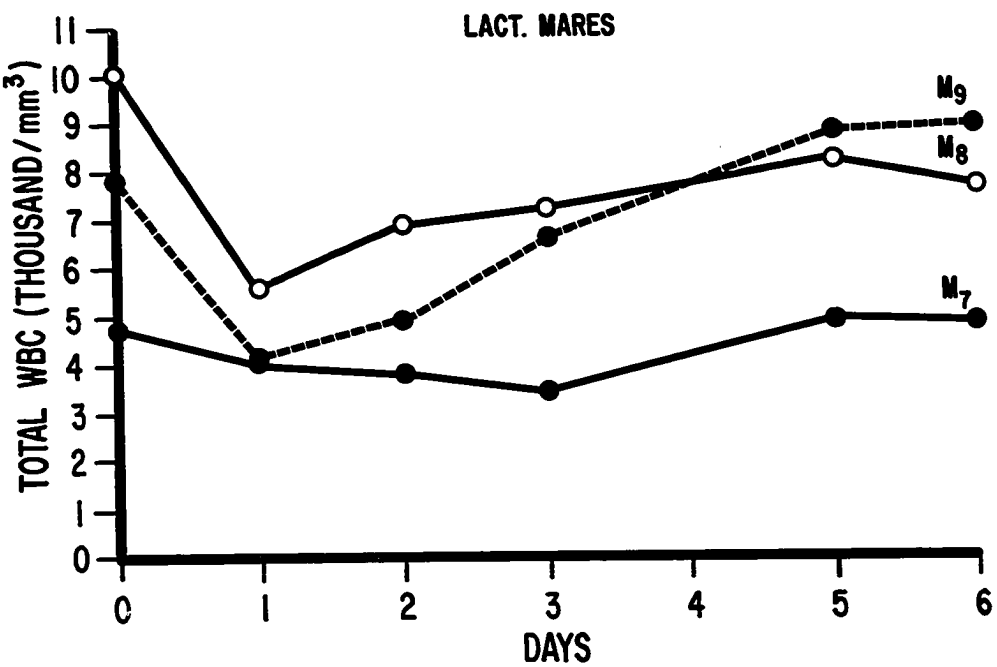

LACT. MARES
TOTAL WBC (THOUSAND/mm³)
11
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
DAYS
M9
M8
M7

Fig. 6. Total white blood cell counts in 3 non-inoculated mares (C1, C2, C3)

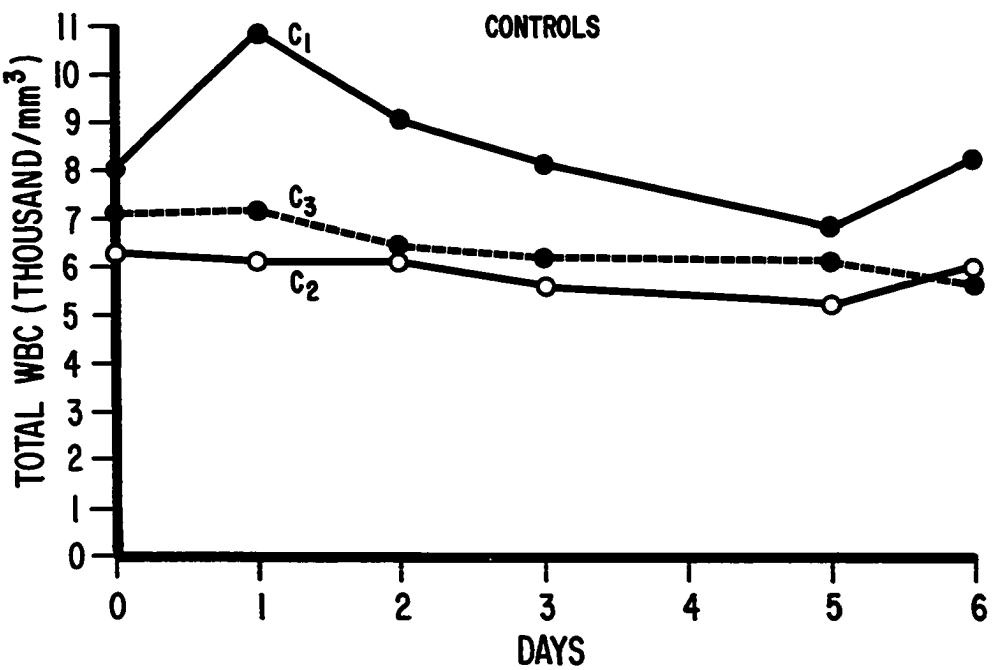

CONTROLS
TOTAL WBC (THOUSAND/mm³)
11
10
9
8
7
6
5
4
3
2
1
0
C1
C3
C2
0
1
2
3
4
5
6
DAYS

Table 1. Neutrophil-lymphocyte (N/L) ratio in peripheral blood of 4 foals (Foals) and 3 mares (Mares) inoculated with equine herpesvirus 1 subtype 2 and in 3 noninoculated mares (Controls)

Days	Foals	Mares	Controls
0[a]	0.45 ± 0.13[b]	1.46 ± 0.36	1.33 ± 0.20
1	0.65 ± 0.09	2.32 ± 1.18	1.90 ± 0.32
2	0.56 ± 0.05	1.98 ± 0.73	1.80 ± 0.29
3	1.50 ± 0.25	1.45 ± 0.19	1.90 ± 0.32
4	-	-	-
5	0.83 ± 0.25	0.79 ± 0.19	1.65 ± 0.08
6	0.96 ± 0.27	0.71 ± 0.05	1.55 ± 0.26

[a]Preinoculation values.

[b]Mean ± standard error of the mean.

after inoculation where it was less than 1 in inoculated mares (Table 1). Hematocrit values were not significantly modified after inoculation in any pony.

A 4-fold increase in VN titer was seen in inoculated mare M8 20 days after inoculation and 43 days after inoculation (Table 2) in inoculated mare M7. In our test conditions, antibody titers of 4 or under were considered as negative and a 4-fold increase in VN titers as a positive response to virus exposure. In sera from foals, all VN titers remained under the limit of detection for our test procedure during the observation period. Serum titers greater than 4 were consistently found in sera from 1 non-inoculated horse (C3).

Virus was not recovered from the blood of any horse at any time. No cytopathogenic effect was seen in EEK cells exposed to blood clots or, on second passage, to first passage cell culture supernatant fluid.

Table 2. Virus neutralization titers in mares (M7-M9) and foals (F6-F9) inoculated with equine herpesvirus 1 subtype 2 and in non-inoculated mares (C1-C3). Titers = reciprocal of serum dilutions giving 100% inhibition of CPE

Horses	0[a]	1	2	3	5	6	20	43
M7	4	4	8	4	4	4	8	16
M8	4	8	8	-	4	4	16	16
M9	4	<4	<4	4	<4	4	4	8
F6	<4	<4	<4	<4	<4	<4	<4	<4
F7	<4	<4	<4	<4	<4	<4	<4	<4
F8	<4	<4	<4	<4	<4	<4	<4	<4
F9	<4	<4	<4	<4	<4	<4	<4	<4
C1	4	4	4	<4	4	4	4	4
C2	4	<4	4	<4	<4	<4	<4	<4
C3	8	8	4	16	8	8	16	16

[a]Preinoculation value for horses of M and F groups.

DISCUSSION

Our studies have shown that strain 76-10356 of EHV-1 subtype 2 is a mild pathogen for adult and foal ponies, and does not elicit overt respiratory disease. Other strains of EHV-1 subtype 2 may be similar. There are indications that inapparent infection may be common with EHV-1 subtype 2 because there are inapparent carriers of EHV-1 (7), and because the respiratory disease is not seen after experimental inoculation with other strains of EHV-1 (2). It is also possible that secondary bacterial infections are responsible for most of the respiratory signs attributed to this virus, as suspected in previous studies (90). In contrast, respiratory disease seen in foals inoculated with subtype 1 virus (117, 118) or subtype 2 virus (119) isolated from aborted fetus (7) may have led to the impression that EHV-1 subtype 2 is a more severe pathogen.

Lack of detectable VN antibodies in sera from inoculated foals may indicate a different immune response in foals compared to pony mares. In contrast with adult horses, foals exposed to EHV-1 rarely develop detectable levels of circulating antibodies as our and other studies have shown (8, 11, 12). However, foals develop a cell-mediated immune (CMI) response as indicated by lymphocyte blastogenesis studies (8). As protection against EHV-1 depends largely on CMI (8, 10, 120), lack of detectable VN antibodies is not sufficient to explain the susceptibility of foals to EHV-1 infections. Other mechanisms of defense of the foal may be deficient in controlling infections. In particular, neutrophils

from foals, compared with neutrophils from adult horses, have reduced motility[e] and, after inoculation with EHV-1 subtype 2, transiently depressed virucidal activity.[f] These characteristics of their neutrophils may partially explain the increased susceptibility of foals to viral and secondary bacterial infections.

The low VN antibody titers of inoculated ponies in this study probably relate to the low pathogenicity of the virus. In contrast with abortigenic strains, respiratory strains of EHV-1 are believed to multiply in the respiratory tract only, without systemic invasion (6, 118). A local infection of the respiratory mucosa is not likely to elicit high antibody titers in the blood. However, the low VN titers may not accurately reflect the level of circulating antibodies. Our VN procedure used EHV-1 subtype 1 as challenge virus. Subtype 1, easier to grow and more reliable in cell cultures than subtype 2, is used in most VN assays for EHV-1 (14). Cross-neutralization between the 2 subtypes may give lower titers against the heterologous subtype. This has been suggested previously (110, 118) but disputed by others using sera from EHV-1 subtype 2 infected gnotobiotic foals (121). Complement might also be needed for detection of antibodies against EHV-1 in sera from foals negative by procedures using heated serum (116).

Failure to isolate the virus from the blood of inoculated ponies indicates that viremia may not occur in EHV-1 subtype 2 infections.

---

[e]F. L. Coignoul et al., Am. J. Vet. Res., in press.

[f]F. L. Coignoul et al., Vet. Pathol., submitted for publication.

Other procedures of virus recovery using washed leukocytes also failed to demonstrate the presence of virus in the blood of infected foals (6).

Leukocytosis and neutrophilia seen after leukopenia in our foals may be due to secondary bacterial infections. Both leukocytosis and neutrophilia are uncommon in viral diseases but are a classical component of bacterial infections. Because of the absence of left shift during neutrophilia, a possible effect of corticosteroids should also be considered. In horses, however, neutrophilia and modified N/L ratio due to corticosteroids is a transient phenomenon (122).

Initial leukopenia in all inoculated ponies, and neutropenia lasting longer than lymphopenia in inoculated mares as shown by the decreased N/L ratio 4 and 5 days after inoculation differ from previous reports (117). Leukopenia appears to be a specific sign of the disease and is not the result of stress. Stress leukogram of horses is characterized by initial lymphocytosis and secondary lymphopenia (122). The higher N/L ratio of foals compared to adult horses is consistent with previous descriptions (123).

GENERAL SUMMARY AND DISCUSSION

The main conclusion of this work is that age-related neutrophil deficiencies may be involved in the high susceptibility of foals to infections. In addition, infection with mild pathogens such as EHV-1 subtype 2 elicits a low burst of activity in foal neutrophils that may allow easier seeding of virus and faster progression of lesions than in adult horses. These conclusions are based on data presented in three separate chapters of this dissertation.

In the first chapter, it was reported that neutrophils from foals were significantly less motile than neutrophils from mares. This low motility can impair an essential step of inflammation and host defense: Migration of phagocytes at the site of injury (124). At the same time, it was seen that other neutrophil functions were somewhat lower in foals than in mares. Morphologically, neutrophil primary granules that contain the enzymes responsible for virucidal activity were partially degranulated in foals and lactating mares, as seen by the increased number of flocculent matrix granules.

In the second study, inoculation of mares and foals with EHV-1 subtype 2 stimulated neutrophil migration. However, migration returned earlier to preinoculation levels in inoculated foals than in inoculated mares. In addition, ADCC, a neutrophil function associated with virucidal activity (16, 17), was reduced in foals after experimental inoculation. More degranulation of primary granules and flocculent matrix granules was consistently seen in neutrophils from inoculated mares than

in foals indicating more intense release of primary granule virucidal enzymes in inoculated mares than in inoculated foals.

In the third chapter, the clinical response of horses to EHV-1 subtype 2 was examined. Fever and leukopenia, more severe in foals than in mares, were the only signs seen after inoculation. Foals did not develop VN antibodies and did not have respiratory signs. These observations are consistent with the effects of a mildly pathogenic virus with local spread that may predispose to secondary bacterial infections.

In this research, some aspects of defense mechanisms in young and mature horses were compared to better understand the susceptibility of foals to infectious agents. In summary: a) impaired motility of foal neutrophils; b) low responsiveness of neutrophils in foals inoculated with EHV-1 subtype 2; and c) low pathogenicity of EHV-1 subtype 2 for horses.

For the future, the goal is to compare major defense mechanisms in young and adult animals of various domestic species and to use this information in prophylaxis and treatment of infections. For now, in vivo evaluation of neutrophil functions in foals should be undertaken.

I am aware of the gap that separates collection of data in test tubes and generation of theoretical hypotheses regarding pathogenesis. However, if I have to face criticisms for making conclusions that go beyond the facts, I will seek shelter in the words of the man who said, "It is true that I have freely put myself among ideas which cannot be rigorously proved. That is my way of looking at things" (Louis Pasteur, quoted by Beveridge) (125).

LITERATURE CITED

1. Hartley, W. J., and R. J. Dixon. An outbreak of foal perinatal mortality due to equid herpesvirus type 1: Pathological observations. Equine Vet. J. 11:215-218, 1979.

2. Prickett, M. E. The pathology of disease caused by equine herpesvirus 1. Pages 24-33 in J. T. Bryans and H. Gerber, eds. Equine infectious diseases II. S. Karger, Basel, Switz. 1970.

3. Bryans, J. T., T. W. Swerczek, R. W. Darlington, and M. N. Crowe. Neonatal foal disease associated with perinatal infection by equine herpesvirus 1. Equine Vet. J. 1:20-26, 1977.

4. Jackson, T. A., B. I. Osburn, D. R. Cordy, and J. W. Kendrick. Equine herpesvirus 1 infection in horses: Studies on the experimentally induced neurologic disease. Am. J. Vet. Res. 38:709-719, 1977.

5. Coggins, L. E. Viral respiratory disease. Vet. Clin. N. Am. 1: 59-72, 1979.

6. Patel, J. R., N. Edington, and J. A. Mumford. Variation in cellular tropism between isolates of equine herpesvirus 1 in foals. Arch. Virol. 74:41-51, 1982.

7. Campbell, T. M., and M. J. Studdert. Equine herpesvirus type 1 (EHV-1). Vet. Bull. 53:135-146, 1983.

8. Gerber, J. D., A. E. Marron, E. P. Bass, and W. H. Beckenhauer. Effect of age and pregnancy on the antibody and cell-mediated immune responses of horses to equine herpesvirus 1. Can. J. Comp. Med. 41: 471-478, 1977.

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

10. Pachciarz, J. A., and J. T. Bryans. Cellular immunity to equine rhinopneumonitis virus in pregnant mares. J. Equine Med. Surg. Suppl. 1:115-128, 1978.

11. Frymus, T. Humoral and cell-mediated immune response of foals vaccinated with attenuated equine herpesvirus type 1 (EHV-1). Zentralbl. Veterinärmed. Reihe B 27:742-758, 1980.

12. Dutta, S. K., and W. D. Shipley. Immunity and the level of neutralization antibodies in foals and mares vaccinated with a modified live-virus rhinopneumonitis vaccine. Am. J. Vet. Res. 36:445-448, 1975.

13. Dutta, S. K., and D. L. Campbell. Cell-mediated immunity in equine herpesvirus type 1 infection. I. In vitro lymphocyte blastogenesis and serum neutralization antibody in normal parturient and aborting mares. Can. J. Comp. Med. 41:404-408, 1977.

14. Doll, E. E., and J. T. Bryans. Development of complement-fixing and virus-neutralizing antibodies in viral rhinopneumonitis of horses. Am. J. Vet. Res. 23:843-846, 1962.

15. Belding, M. E., and S. J. Klebanoff. Peroxidase-mediated virucidal systems. Science 16:195-196, 1970.

16. Russel, A. S., and C. Miller. A possible role for polymorphonuclear leukocytes in the defence against recrudescent herpes simplex virus infection in man. Immunology 34:371-378, 1978.

17. Rouse, B. T., R. C. Wardley, and L. A. Babiuk. Antibody-dependent cell-mediated cytotoxicity in cows: Comparison of effector cell activity against heterologous erythrocyte and herpesvirus-infected bovine target cells. Infect. Immun. 13:1433-1441, 1976.

18. Jubb, K. V. F., and P. C. Kennedy. Influenza and other viral respiratory infections of horses including equine viral rhinopneumonitis. Pages 214-215 in K. V. F. Jubb and P. C. Kennedy, eds. Pathology of domestic animals, 2nd ed. Academic Press, New York, N.Y. 1970.

19. Jones, T. C., and R. D. Hunt. Equine viral rhinopneumonitis. Pages 332-336 in T. C. Jones and R. D. Hunt, eds. Veterinary pathology, 5th ed. Lea and Febiger, Philadelphia, PA. 1983.

20. Murphy, P. The neutrophil - Introduction. Pages 1-5 in P. Murphy, ed. The neutrophil. Plenum Medical Book Company, New York, N.Y. 1976.

21. Cornely, H. P. Reversal of chemotaxis in vitro and chemotactic activity of leukocyte fractions. Proc. Soc. Exp. Biol. Med. 122:831-835, 1966.

22. Wilson, A. T., G. G. Wiley, and P. Bruno. Fate of non virulent streptococci phagocytized by human and mouse neutrophils. J. Exp. Med. 106:777-786, 1957.

23. Klebanoff, S. J. Iodination of bacteria: A bactericidal mechanism. J. Exp. Med. 126:1063-1079, 1967.

24. Sbarra, A. J., and M. L. Karnovsky. The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. J. Biol. Chem. 234:1355-1362, 1959.

25. Harris, P. F., and J. H. Kluger. Mitosis in metamyelocyte. Nature 200:712-713, 1963.

26. Boggs, D. R. The kinetics of neutrophilic leukocytes in health and disease. Semin. Hematol. 4:359-386, 1967.

27. Bainton, D. F., and M. G. Farquhar. Origin and nature of polymorphonuclear leukocyte granules. J. Cell Biol. 27:6A, 1965.

28. Bainton, D. F., and M. G. Farquhar. Origin of granules in polymorphonuclear leukocytes. Two types derived from opposite faces of the Golgi complex in developing granulocytes. J. Cell Biol. 28:277-301, 1966.

29. Bainton, D. F., and M. G. Farquhar. Differences in enzyme content of azurophilic and specific granules of polymorphonuclear leukocytes (I). J. Cell Biol. 39:286-298, 1968.

30. Anderson, D. C., L. J. Wible, B. J. Hughes, C. W. Smith, and B. R. Brinkley. Cytoplasmic microtubules in polymorphonuclear leukocytes: Effects of chemotactic stimulation and colchicine. Cell 31: 719-729, 1982.

31. Hoffstein, S., I. M. Goldstein, and G. Weissmann. Role of microtubule assembly in lysosomal enzyme secretion from human polymorphonuclear leukocytes. J. Cell Biol. 73:242-256, 1977.

32. Oliver, J. M. Cell biology of leucocyte abnormalities. Membrane and cytoskeletal function in normal and defective cells. Am. J. Pathol. 93:221-259, 1978.

33. Zurier, R. B., G. Weissmann, S. Hoffstein, S. Kammerman, and H. H. Tai. Mechanisms of lysosomal enzyme release from human leukocytes (II). J. Clin. Invest. 53:297-309, 1974.

34. Rudolph, S. A., P. Greengard, and S. E. Malawista. Effects of colchicine on cyclic AMP levels in human leukocytes. Proc. Nat. Acad. Sci. USA 74:3404-3408, 1977.

35. Gallin, J. I. Abnormal phagocyte chemotaxis: Pathophysiology, clinical manifestations, and management of patients. Rev. Infect. Dis. 3:1196-1213, 1981.

36. Slauson, D. O. Cellular events in inflammation. Pages 168-182 in D. O. Slauson and B. J. Cooper, eds. Mechanisms of disease. Williams and Wilkins, Baltimore, MD. 1982.

37. Stryer, L. Protein synthesis in bacteria is initiated by formylmethionine transfer RNA. Pages 656-657 in L. Stryer, ed. Biochemistry, 2nd ed. W. H. Freeman and Company, San Francisco, CA. 1981.

38. Williams, L. E., R. Snyderman, M. C. Pike, and R. J. Lefkowitz. Specific receptor sites for chemotactic peptides on human polymorphonuclear leukocytes. Proc. Nat. Acad. Sci. USA 74:1204-1208, 1977.

39. Smith, C. W. and J. C. Hollers. Motility and adhesiveness in human neutrophils. J. Clin. Invest. 65:804-812, 1980.

40. Zigmond, S. H. Mechanisms of sensing chemical gradients by polymorphonuclear leukocytes. Nature 249:450-452, 1974.

41. Gallin, J. I., D. G. Wright, H. L. Malech, J. M. Davis, M. S. Klempner, and C. H. Kirkpatrick. Disorders of phagocyte chemotaxis. Ann. Int. Med. 92:520-538, 1980.

42. Schliwa, M., K. B. Pryzwansky, and U. Eutenever. Centrosome splitting in neutrophils: An unusual phenomenon related to cell activation and motility. Cell 31:705-717, 1982.

43. Malech, H. L., R. K. Root, and J. I. Gallin. Structural analysis of human neutrophil migration: Centriole, microtubule and microfilament orientation and function during chemotaxis. J. Cell Biol. 75:666-693, 1977.

44. Burchill, B. R., J. M. Oliver, C. B. Pearson, E. D. Leinbach, and R. D. Berlin. Microtubule dynamics and glutathione metabolism in phagocytizing human polymorphonuclear leucocytes. J. Cell Biol. 76:439-447, 1978.

45. DeBrabander, M. Microtubules, central elements of cellular organization. Endeavour 6:124-134, 1982.

46. Wright, D. G., and J. I. Gallin. Secretory response of human neutrophils: Exocytosis of specific (secondary) granules by human neutrophils during adherence in vitro and during exudation in vivo. J. Immunol. 123:285-294, 1979.

47. Horwitz, M. A. Phagocytosis of microorganisms. Rev. Infect. Dis. 4:104-123, 1982.

48. Root, R. K., and T. P. Stossel. Myeloperoxidase-mediated iodination by granulocytes. J. Clin. Invest. 53:1207-1215, 1974.

49. Weissmann, G., J. E. Smolen, and H. M. Korchak. Release of inflammatory mediators from stimulated neutrophils. N. Engl. J. Med. 303:27-34, 1980.

50. Fantone, J. C., and P. A. Ward. Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. Am. J. Pathol. 107:397-417, 1982.

51. Klebanoff, S. J., and R. A. Clark. Iodination by human polymorphonuclear leukocytes: A reevaluation. J. Lab. Clin. Med. 89: 675-686, 1977.

52. Weissmann, G., P. Dukor, and R. B. Zurier. Effect of cyclic AMP on release of lysosomal enzymes from phagocytes. Nat. New Biol. 231: 131-135, 1971.

53. Bagby, G. C., and R. M. Bennett. Feedback regulation of granulopoiesis: Polymerization of lactoferrin abrogates its ability to inhibit CSA production. Blood 60:108-112, 1982.

54. Gimbrone, M. A. Blood vessels and the new mediators of inflammation. Lab. Invest. 46:454-455, 1982.

55. Svehag, S. E. Formation and dissociation of virus-antibody complexes with special reference to the neutralization process. Pages 1-63 in J. L. Melnick, ed. Progress in medical virology, Vol. 10. S. Krager, Basel, Switz. 1968.

56. Gresser, I., and D. J. Lang. Relations between viruses and leukocytes. in J. L. Melnick, ed. Progress in medical virology, Vol. 8. Hafner Publishing Company, Inc., New York, N.Y. 1966.

57. Sommerville, R. G., and P. S. McFarlane. The rapid diagnosis of virus infections by immunofluorescent techniques applied to blood leukocytes. Lancet 1:911-912, 1964.

58. McKercher, D. G., J. K. Saito, and R. M. Mathis. Comparative aspects of immunity against bovine and equine herpesviruses. J. Am. Vet. Med. Assoc. 155:300-306, 1969.

59. Rouse, B. T., L. A. Babiuk, and P. M. Henson. Neutrophils in antiviral immunity: Inhibition of virus replication by a mediator produced by bovine neutrophils. J. Infect. Dis. 141:223-232, 1980.

60. Smith, J. W., and A. M. Sheppard. Activity of rabbit monocytes, macrophages, and neutrophils in antibody-dependent cellular cytotoxicity of herpes simplex virus-infected corneal cells. Infect. Immun. 36:685-690, 1982.

61. Gale, R. P., and J. Zighelboim. Polymorphonuclear leukocytes in antibody-dependent cellular cytotoxicity. J. Immunol. 114:1047-1051, 1975.

62. Lopez, A. F., and C. J. Sanderson. Antibody-dependent cell-mediated cytotoxicity of nucleated mammalian cells by rat eosinophils and neutrophils. Int. Arch. Allergy Appl. Immunol. 67:200-205, 1982.

63. Grewal, A. S., B. T. Rouse, and L. A. Babiuk. Mechanisms of resistance to herpesviruses: Comparison of the effectiveness of different cell types in mediating antibody dependent cell mediated cytotoxicity. Infect. Immun. 15:698-703, 1977.

64. Fujimiya, Y., B. T. Rouse, and L. A. Babiuk. Human neutrophil-mediated destruction of antibody sensitized herpes simplex virus type 1 infected cells. Can. J. Microbiol. 24:182-186, 1978.

65. Siebens, H., S. S. Tevethia, and B. M. Babior. Neutrophil-mediated antibody-dependent killing of herpes-simplex-virus-infected cells. Blood 54:88-93, 1979.

66. Shore, S. S., and T. J. Romano. Analysis of the lytic step in the herpes simplex virus antibody-dependent cellular cytotoxicity system. Infect. Immun. 28:137-146, 1980.

67. Grewal, A. S., M. Carpio, and L. A. Babiuk. Polymorphonuclear neutrophil-mediated antibody-dependent cell cytotoxicity of herpesvirus-infected cells: Ultrastructural studies. Can. J. Microbiol. 26:427-435, 1980.

68. Grewal, A. S., and L. A. Babiuk. Complement-dependent polymorphonuclear neutrophil-mediated cytotoxicity of herpesvirus-infected cells: Possible mechanism(s) of cytotoxicity. Immunology 40:151-161, 1980.

69. Sonoda, M., and K. Kobayashi. Electron microscopic observations on the blood of the horse. I. Neutrophils in the peripheral blood of clinically healthy horses. Jpn. J. Vet. Res. 14:71-85, 1966.

70. Bertram, T. A., and F. L. Coignoul. Morphometry of equine neutrophils isolated at different temperatures. Vet. Pathol. 19:534-543, 1982.

71. Bertram, T. A., F. L. Coignoul, and A. E. Jensen. Phagocytosis and intra-cellular killing of the contagious equine metritis organism by equine neutrophils in serum. Infect. Immun. 37:1241-1247, 1982.

72. Bertram, T. A., F. L. Coignoul, and A. E. Jensen. Phagocytosis and intra-cellular killing of contagious equine metritis organism by equine neutrophils in genital secretions. Am. J. Vet. Res. 44:(in press), 1983.

73. Jacobsen, K., S. M. Reed, J. Newbry, W. M. Baily, L. E. Perryman, and R. W. Leid. Isolation of equine neutrophils and analysis of functional characteristics by chemiluminescence and bactericidal assays. Am. J. Vet. Res. 43:1912-1916, 1982.

74. Camp, C. J., and R. W. Leid. Chemotaxis of radiolabeled equine neutrophils. Am. J. Vet. Res. 43:397-401, 1982.

75. Fujimiya, Y., L. E. Perryman, and T. B. Crawford. 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.

76. McGuire, T. C., L. E. Perryman, T. B. Crawford, and J. R. Gorham. Characterization of the interaction of IgG(T) with the group specific antigen P28 of equine infectious anemia virus in immunodiffusion and complement-fixation tests. J. Equine Med. Surg. Suppl. 1:375-380, 1978.

77. Dimock, W. W., and P. R. Edwards. The differential diagnosis of equine abortion with special reference to a hitherto undescribed form of epizootic abortion in mares. Cornell Vet. 26:231-240, 1936.

78. McGee, W. R. Clinical aspects of disease caused by equine herpesvirus 1. Pages 13-17 in J. T. Bryans and H. Gerber, eds. Equine infectious diseases II. S. Karger, Basel, Switz. 1970.

79. Dimock, W. W. Diagnosis of virus abortion in mares. J. Am. Vet. Med. Assoc. 106:665-666, 1940.

80. Manninger, R., and J. Czontos. Virusabortus der Stuten. Dtsch. Tierärztl. Wochenschr. 49:104-108, 1941.

81. Doll, E. R. Intrauterine and intrafetal inoculations with equine abortion virus in pregnant mares. Cornell Vet. 43:112-121, 1953.

82. Doll, E. R., J. T. Bryans, W. H. McCollum, and M.E.W. Crowe. Isolation of a filterable agent causing arteritis in horses and abortion in mares. Its differentiation from the equine abortion (influenza) virus. Cornell Vet. 47:3-14, 1957.

83. Plummer, G., and A. P. Waterson. Equine herpesviruses. Virology 19:412-416, 1963.

84. Studdert, M. J. Comparative aspects of equine herpesviruses. Cornell Vet. 64:94-122, 1974.

85. Burrows, R., and D. Goodridge. In vivo and in vitro studies of equine rhinopneumonitis virus strains. Pages 306-321 in J. T. Bryans and H. Gerber, eds. Equine infectious diseases III. S. Karger, Basel, Switz. 1973.

86. Howe, C., J. E. Coward, and T. W. Fenger. Viral invasion: Morphological, biochemical, and biophysical aspects. Pages 54-63 in H. Fraenkel-Conrad and R. R. Wagner, eds. Comprehensive virology 16. Plenum Press, New York, N.Y. 1980.

87. Abodeely, R. A., L. A. Lawson, and C. C. Randall. Morphology and entry of enveloped and deenveloped equine abortion (herpes) virus. J. Virol. 5:513-523, 1970.

88. Dales, S. Early events in cell-animal virus interactions. Bacteriol. Rev. 37:103-135, 1973.

89. Kendrick, J. W. Comments on equine herpesvirus infection (equine rhinopneumonitis) and bovine herpesvirus infection (infectious bovine rhinotracheitis). J. Am. Vet. Med. Assoc. 155:306-309, 1969.

90. Thomson, G. R., J. A. Mumford, and W. Plowright. Immunological responses of conventional and gnotobiotic foals to infectious and inactivated antigens of equine herpesvirus 1. J. Equine Med. Surg. Suppl. 1:113-114, 1978.

91. Osburn, B. I., G. H. Stabenfeldt, A. A. Ardans, C. Trees, and M. Sawyer. Perinatal immunity in calves. J. Am. Vet. Med. Assoc. 164:295-298, 1974.

92. Cheville, N. F. The gray collie syndrome. J. Am. Vet. Med. Assoc. 152:620-630, 1968.

93. Quie, P. G. Infections in patients with abnormal granulocyte chemotaxis. Springer Semin. Immunopathol. 4:241-252, 1981.

94. Roth, J. A., and M. L. Kaeberle. Evaluation of bovine polymorphonuclear leukocyte function. Vet. Immunol. Immunopathol. 2:157-174, 1981.

95. Roth, J. A., and M. L. Kaeberle. Isolation of neutrophils and eosinophils from the peripheral blood of cattle and comparison of their functional activities. J. Immunol. Meth. 45:153-164, 1981.

96. Al Nakeeb, S., and E. N. Thompson. Assessment of neutrophil chemotaxis and random migration in childhood. Arch. Dis. Childh. 55: 296-298, 1980.

97. Klein, R. B., T. J. Fisher, S. E. Gard, M. Biberstein, K. C. Rich, and E. R. Stiehm. Decreased mononuclear and polymorphonuclear chemotaxis in human newborns, infants, and young children. Pediatrics 60:467-472, 1977.

98. Miller, M. E. Phagocytic function in the neonate: Selected aspects. Pediatrics 64:709-712, 1979.

99. Krause, P. J., E. G. Maderazo, and M. Scroggs. Abnormalities of neutrophil adherence in newborns. Pediatrics 69:184-187, 1982.

100. Anderson, D. C., L. K. Pickering, and R. D. Feigin. Leukocyte function in normal and infected neonates. J. Pediatr. 85:420-425, 1974.

101. Murphy, S., and D. E. Van Epps. Neutrophil and monocyte function in pediatric patients with recurrent pneumonias. Am. Rev. Resp. Dis. 126:92-96, 1982.

102. Bjorksten, B. Phagocyte function in pregnancy. Immunol. Today 1:55-56, 1980.

103. Colley, D. G., and S. L. James. Participation of eosinophils in immunological systems. Pages 55-85 in M. D. Gupta and R. A. Good, eds. Cellular, molecular, and clinical aspects of allergic disorders. Plenum Press, New York, NY. 1979.

104. Oleske, J. M., R. B. Ashman, S. Kohl, S. L. Shore, S. E. Starr, P. Wood, and A. J. Nahmias. Human polymorphonuclear leukocytes as mediators of antibody-dependent cell mediated cytotoxicity to herpes simplex virus infected cells. Clin. Exp. Immunol. 27:446-453, 1977.

105. Rouse, B. T., R. C. Wardley, L. A. Babiuk, and T.K.S. Mukkur. The role of neutrophils in antiviral defense. *In vitro* studies on the mechanism of antiviral inhibition. J. Immunol. 118:1957-1961, 1977.

106. Carlson, G. P., and J. J. Kaneko. Intravascular granulocyte kinetics in developing calves. Am. J. Vet. Res. 36:421-425, 1975.

107. Estensen, R. D., J. G. White, and B. Holmes. Specific degranulation of human polymorphonuclear leukocytes. Nature 248:347-348, 1974.

108. Silverstein, S. C., R. M. Steinman, and Z. A. Cohn. Endocytosis. Ann. Rev. Biochem. 46:669-722, 1977.

109. O'Callaghan, D. J., G. P. Allen, and C. C. Randall. Structure and replication of the equine herpesviruses. J. Equine Med. Surg. Suppl. 1:1-31, 1978.

110. Horner, G. W. Serological relationship between abortifacient and respiratory strains of equine herpesvirus type 1 in New Zealand. N. Z. Vet. J. 29:7-8, 1981.

111. Studdert, M. J. Differentiation of respiratory and abortigenic isolates of equine herpesvirus 1 by restriction endonucleases. Science 214:562-564, 1981.

112. Thein, P. Infection of the central nervous system of horses with equine herpesvirus serotype 1. J. S. Afr. Vet. Med. Assoc. 52:239-241, 1981.

113. Allen, G. P., M. R. Yeargan, L. W. Turtinen, J. T. Bryans, and W. H. McCollum. Molecular epizootiologic studies of equine herpesvirus-1 infections by restriction endonuclease fingerprinting of viral DNA. Am. J. Vet. Res. 44:263-271, 1983.

114. Turtinen, L. W., G. P. Allen, R. W. Darlington, and J. T. Bryans. Serologic and molecular comparisons of several equine herpesvirus type 1 strains. Am. J. Vet. Res. 42:2099-2104, 1981.

115. Dutta, S. K., A. Myrup, and M. K. Baumgardner. Lymphocyte responses to virus and mitogen in ponies during experimental infection with equine herpesvirus 1. Am. J. Vet. Res. 41:2066-2068, 1980.

116. Snyder, D. B., A. C. Myrup, and S. K. Dutta. Complement requirement for virus neutralization by antibody and reduced complement levels associated with experimental equine herpesvirus 1 infection. Inf. Immun. 31:636-640, 1981.

117. Turner, A. J., M. J. Studdert, and J. E. Peterson. Equine herpesviruses. 2. Persistence of equine herpesviruses in experimentally infected horses and the experimental induction of abortion. Aust. Vet. J. 46:90-98, 1970.

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

119. Kawakami, Y., and T. Shimizu. Combined immunizing effects of live and inactivated equine herpesvirus type 1 in horses. J. Equine Med. Surg. Suppl. 1:75-82, 1978.

120. Wilks, C. R., and L. Coggins. An assessment of lymphocyte transformation and cytotoxicity following infection with equine herpesvirus type 1. J. Equine Med. Surg. Suppl. 1:93-101, 1978.

121. Thomson, G. R., J. A. Mumford, J. Campbell, L. Griffiths, and P. Clapham. Serological detection of equid herpesvirus 1 infections of the respiratory tract. Equine Vet. J. 8:58-65, 1976.

122. Rossdale, P. D., P. N. Burguez, and R. S. G. Cash. Changes in blood neutrophil/lymphocyte ratio related to adrenocortical function in the horse. Equine Vet. J. 14:293-298, 1982.

123. Sato, T., K. Oda, and M. Kubo. Hematological and biochemical values of thoroughbred foals in the first six months of life. Cornell Vet. 69:3-19, 1978.

124. Larsen, G. L., and M. Henson. Mediators of inflammation. Ann. Rev. Immunol. 1:335-359, 1983.

125. Beveridge, W.I.B. The art of scientific investigation. Page 200 in W.I.B. Beveridge, ed. The art of scientific investigation. Vintage books, New York, N.Y. 1950.

ACKNOWLEDGMENTS

Proper thanks to all those people that for 4 years have given to me their time and their skills is impossible. A list would necessarily be incomplete and thus unfair. Therefore, I will limit my words to those that day after day made my work easier and my life more enjoyable.

My supervisor, Dr. Norman Cheville, was from the start the example to follow. From him, I gained the insight of what it takes to become a pathologist and a scientist. The privilege of having worked with this exceptional man is a heritage that I pride.

The enthusiasm and intelligence of Dr. Timothy Bertram stimulated me to become a better scientist, a better man, ...and a better American. His friendship is a gift that time and distance will not alter or erase.

From my other friends in the laboratory, and from those in our supply unit across the hall, I received the best possible help, all made of kindness and dedication. They all deserve my heartfelt acknowledgments.

I am deeply indebted to the Director of the National Animal Disease Center where I worked and to the librarians, animal supply personnel, and clerical staff that helped bring my research, my publications, and my dissertation into their present form.

Finally, I wish to dedicate this dissertation to a great and generous country: the United States of America.