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CHAPTER 2

MECHANISMS OF INFECTIOUS DISEASE

2.1—Mechanisms of Establishment and Spread of Bacterial and Fungal Infections

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Normal Flora

Dermal and mucosal surfaces provide a life-preserving protective barrier composed of physical, chemical, and microbial defenses. Normal flora contributes to this protective barrier against pathogens while paradoxically providing a source for potential opportunistic invasion. Commensal bacteria are those that benefit from living on or within a host without mediating harm. For a relationship to be commensal, it must be mutually beneficial, and the interruption of this association results in abnormal host development or overt disease.[1] A pathogen is any disease-producing organism; thus a commensal organism has the potential to be pathogenic. Colonization is infection without disease. Skin, gastrointestinal, respiratory, and urogenital colonization occurs early in life and persists unless disrupted.

SKIN FLORA

The combination of normal flora and mucosal immunity provides an effective barrier against infectious colonization of nondisrupted skin surfaces. Specific bacteria are stratified by site, and certain bacteria multiple and colonize depending on associated adnexa.[2] Most bacteria and fungi on the surface of the skin are not associated with disease; however, yeast and bacteria within hair follicles are more likely to be related to a disease process.[3] Even though horses inhabit an environment heavily contaminated with fecal flora, normal dermal flora in the horse is surprisingly devoid of members of the Enterobacteriaceae.[4] Normal inhabitants include mixed populations of bacteria of species of *Acinetobacter, Aerococcus, Aeromonas, Bacillus, Corynebacterium, Flavobacterium, Micrococcus, Nocardia,* coagulase-negative *Staphylococcus, Staphylococcus aureus, Streptomyces,* and nonhemolytic *Streptococcus* generae.[2] Certain *Staphylococcus* spp. have been associated with skin disease in the horse and these include *S. aureus, S. intermedius,* and *S. hyicus,* whereas species such as *S. xylosus* and *S. sciuri* more often are associated with normal skin.[5] More than 30 species of fungi can inhabit the skin, and *Alternaria, Aspergillus, Candida, Fusarium, Rhizopus,* and *Trichophyton* spp.[2] commonly are present.

ORAL, PHARYNGEAL, AND RESPIRATORY FLORA

Oral and pharyngeal mucosal flora is associated with health and disease of the upper and lower respiratory system. The oral and pharyngeal mucosa is populated richly with many bacteria, including obligate aerobes, anaerobes, and facultative anaerobes.[6] Aerobic and facultative anaerobic populations are comprised mainly of *Streptococcus equi, Pasteurella* spp., *Escherichia coli, Actinomyces* spp., and *Streptococcus* spp. Anaerobes actually predominate in the mouth of the normal horse, and colonization of the oropharynx and mouth consists of members of several bacterial genera. Gram-positive and gram-negative anaerobic species inhabit the pharyngeal tonsillar area, and *Bacteroides fragilis* and *Bacteroides* spp. predominate. *Fusobacterium* spp., *Eubacterium* spp., *Clostridium* spp., *Veillonella* spp., and *Megasphaera* spp.

are also common. These same genera are found consistently in horses with lower respiratory infections, indicating that pharyngeal flora is a likely source of contamination.[6] Contamination of the trachea of the horse occurs frequently, as evidenced by the fact that transtracheal aspiration yields positive bacterial cultures in approximately 30% of normal adult horses and foals.[7] As with skin flora, normal horses have multiple fungal species inhabiting conjunctival, nasal, and oral mucosae. Stabling increases the frequency of ocular fungi in normal horses.[8]

INTESTINAL FLORA

In animal models and chronic human conditions, normal flora is considered important for intestinal maturity and containment of disease. Changes in cecal weight, villus-to-crypt ratio, development of gut immunoglobulin A responses, and volatile fatty acid production are affected by suboptimal cecal colonization in germ-free animals.[9] A relationship between severity of mucosal disease and normal flora also has been demonstrated in models of inflammatory bowel disease of human beings.[10]

Bacteria are present in all parts of the intestinal tract of the horse, and the microbial fauna increases in complexity and density aborally.[11] The stomach of the horse is not a sterile environment. A dense population of gram-positive bacterial rods, primarily composed of *Lactobacillus* spp., colonizes the nonsquamous portion of the equine stomach. Substantial colonization of the duodenum occurs with a large population of proteolytic bacteria, and this colonization increases tenfold in the ileum.[12]

Microbial degradation and fermentation of plant material in the large intestine is an important component of nutrient acquisition in the horse. The consumption of cellulose and starch results in the production of volatile fatty acids.[13] The major cellulolytic bacterial species of the large intestine are similar to those of Bovidae, but the strains in the horse produce different arrays of fermentation products.[14] As in cows, *Ruminococcus flavefaciens* is one of the predominate cellulolytic bacteria of the equine cecum. Overall, gram-negative rods are the most populous bacteria, comprising 50% of flora. Gram-positive rods and cocci each represent around 20% of the bacterial population. Predominant species include members of the Enterobacteriaceae, *Butynvibrio* spp., *Streptococcus* spp., *Bacteroides* spp., *Lactobacillus* spp., *Selenomonas* spp., *Eubacterium* spp., *Propionibacterium* spp., *Staphylococcus* spp., and many unclassified rods and cocci.[15] Yeasts and fungi of the order Mucorales have been identified in the cecum of normal horses and are capable of digesting cellulose and starch.[16]

The latest investigations demonstrate a lack of intestinal pathogens in the flora of normal horses as detected by routine culturing of feces. In the largest study to date, fecal shedding of *Salmonella* in normal horses from farms without evidence of salmonellosis was 0.8% in resident horses.[17] Based on limited investigations, the carriage rates of *Clostridium difficile* in normal horses and foals also appears to be low (<1.5%).[18] Intestinal flora in the horse is an important source for extraintestinal pathogens. In studies examining the carriage rate of *Rhodococcus equi,* all horses cultured carried the bacteria regardless of age.[19,20] Furthermore, if the farm had endemic *R. equi* and respiratory isolates contain the 90-kd plasmid, which has been associated with disease, fecal isolates also contained this plasmid.

UROGENITAL FLORA

By far most of the work that characterizes equine normal flora has focused on urogenital flora in an effort to gain understanding of the role of uterine contamination or infection in fetal loss. Although the vaginal and vestibular mucosae of mares are colonized with normal mucosal flora, the uterus is considered sterile. However, typical culturing techniques result in frequent isolation of what might be considered pathogens, and cytologic examination and bacterial counts are important supplemental tests for detecting true infection. Colony counts less than 10 colony-forming units and lack of inflammatory cells indicate uterine or technical contamination.[21] Many bacteria inhabit the external genitalia of stallions, including those considered to be associated with metritis in mares. The predominant aerobe isolated is coagulase-negative *Staphylococcus* spp., followed by *Corynebacterium* spp., α-hemolytic *Streptococcus* spp., and *Lactobacillus* spp. Pathogens such as β-hemolytic *Streptococcus* spp., *Pseudomonas aeruginosa,* and *Klebsiella* spp. can be found frequently in servicing stallions.[22,23] Pregnancy rates appear to be the same in mares bred to stallions with semen infected with *P. aeruginosa*.[24]

FUNGAL FLORA

Essentially the same principles apply regarding normal flora, host immunity, and specific virulence factors for the pathogenesis of fungal infection. Fungal infections can be divided into primary or opportunistic pathogens. True pathogens are less dependent on host status than are opportunistic pathogens, although even a true pathogen may require some degree of alteration of normal flora or host immunity to become established. Long-term antibiotic use, immunosuppression, and compromised organ function especially involving the pulmonary or endocrine system are three primary host factors highly associated with establishment of opportunistic fungal infection. Fungi often can adapt to the mammalian environment over a short time. Adaptation usually requires a change in thermal range, oxygen requirements, and resistance to host defenses.

Population Biology of Bacterial Infections

Inoculum size, virulence of the organism, and microbial resistance are three important determinants in the outcome of infectious challenge. Understanding the importance of inoculum size in bacterial pathogenesis in general and for a particular bacterium has profound implications for prevention and management of disease. Minimizing the size of bacterial challenge by controlling environmental contamination is the ultimate goal of any management strategy for animals in their natural environment and in hospital settings. Control of environmental challenge dose is especially important in the control of nosocomial and iatrogenic disease.

INOCULUM SIZE

Although inoculum size and development of disease are studied frequently in equine viral diseases, comparatively little dose-response work is done to demonstrate a clear relationship between frequency or severity of disease and dose for many equine bacterial pathogens. In general, for systemic infections, inoculum size is the most important determinant of disease in hematogenously disseminated infections such as meningitis and, in rodent models, osteomyelitis.[25,26] Failure of passive transfer, immunodeficiency, infection in multiple joints, and infection with *Salmonella* spp. are important determinants in survival regarding equine neonatal osteomyelitis, but actual data on bacterial characteristics are limited. For skin infections, colonization of experimentally induced wounds with *Staphylococcus* spp. depends on inoculum size.[27] Thus control of surgical and wound hygiene depends on agents to which *Staphylococcus* is sensitive. The occurrence of diarrhea has been demonstrated to be dose-dependent in salmonellosis in calves,[28] but little is known regarding challenge dose in the foal or the adult horse. Minimal inoculum size is required for certain pathogens that form toxins, such as *Escherichia coli* and *Clostridium* spp. For instance, disease in human beings caused by *E. coli* 0157:H7 requires a small inoculum, and other factors such as diet or antibiotics promote colonization of a few organisms, resulting in severe disease.[29]

VIRULENCE

Virulence is the ability of an organism to cause disease.[1] Virulence of an organism frequently is tested by inoculation of different strains of a pathogen into groups of a rodent species and evaluation of lethality or invasiveness. Using this particular methodology, the severity of many diseases frequently is found to be strain-dependent, and virulence commonly is associated with certain phenotypic characteristics of a particular strain. For example, severity of pulmonary disease resulting from *Klebsiella pneumoniae* depends on differences in capsular size and colony morphology.[30] These characteristics become the focus of future investigations and eventually may be defined as virulence factors.

ANTIBIOTIC RESISTANCE

Widespread use of antibiotics in animal and human infection occurred following World War II.[31,32] Within 30 years, resistance of gram-positive organisms already was occurring in human pneumococcal infections.[33] From the 1960s to the 1980s, staphylococcal resistance progressed from initial methicillin-resistant organisms to vancomycin-resistant organisms.[32] As a result, for gram-positive and gram-negative organisms, progressive resistance has resulted in less effective means for therapeutic intervention.

Although specific types and mechanisms of bacterial resistance are discussed elsewhere, high-grade and intermediate resistance occur, which are speculated to have arisen from different processes. In terms of bacterial pathogenesis, intermediate resistance occurs in geographically defined isolates in a stepwise fashion resulting from genetic changes under antibiotic pressure. Thus proper dose and length of antibiotic exposure is important in preventing the development of these isolates.[33,34] High-grade resistance is associated with multidrug resistance.[35] In high-grade and multidrug forms of antibiotic resistance, clonal expansion of small numbers of isolates occurs.[35] Individual carrier animals and persons are important for high-grade and multidrug resistance in addition to selective antibiotic pressure. Of note is the fact that commensal or noninvasive pathogens have higher reportage rates for resistance, whereas more highly pathogenic or invasive organisms have less resistance. Thus virulence and antibiotic resistance are not synonymous. Because commensal organisms are ubiquitous, they have a higher likelihood of contact with bacteria that have multiresistance genes. Thus commensal organisms eventually become a reservoir for resistance genes.[36]

Development of Disease and the Role of Normal Flora

DISRUPTION OF NORMAL FLORA

As stated earlier, development of disease is caused by disruption of normal flora and invasion by a pathogen or the conversion of a common commensal organism into a pathogen. The pathophysiology of certain types of equine colitis and pleuropneumonia provide examples consistent with each of these two circumstances. Disruption of normal gastrointestinal flora is likely the underlying

pathogenic mechanism for development of acute enterocolitis. Development of colitis in the horse, presumably from disruption of fecal flora, has been associated with feed change, antibiotics, surgery, nonsteroidal antiinflammatory drugs, and transport.[37-39] Rapid change from a roughage diet to concentrate results in increased numbers of anaerobes, decreased numbers of cellulolytic bacteria, decreased cecal protozoa diversity, and decreased pH in the equine cecum.[37] Isolation of *Clostridium difficile* increases in horses administered antibiotics, and *C. difficile* diarrhea associated with ampicillin, erythromycin, penicillin, and potentiated sulfonamides has been reported in adult horses.[38,40] In ponies infected with *Salmonella* spp., transport and surgery reactivated infection and diarrhea, and antibiotics (oxytetracycline) prolonged shedding.[39] In a case-control study, potentiated sulfonamides were not associated significantly with development of diarrhea in hospitalized horses; however, overall antibiotic use was associated highly with the occurrence of diarrhea.[41]

Several mechanisms exist by which antibiotics disrupt normal gastrointestinal flora and intestinal function. These mechanisms include disruption of carbohydrate metabolism, decreased metabolism of bile acids, direct effects on intestinal motility, and alterations in intestinal mucosa. Change in carbohydrate metabolism is a large intestinal event following decreased microbial reduction of carbohydrates to short-chain fatty acids (SCFA). Because SCFA metabolism and absorption results in fluid and electrolyte absorption, a sudden decrease in SCFAs leads to osmotic diarrhea with intraluminal accumulation of organic acids, cations, and carbohydrates. In human studies, reduced SCFAs have been demonstrated with many antibiotics, including ampicillin, metronidazole, and erythromycin. Bile acids, reduced in the colon by dehydroxylating bacteria, are potent colonic secretogues. Increases in fecal bile acids have been demonstrated in human beings with the use of ampicillin and clindamycin. Erythromycin and amoxicillin directly affect colonic motility.[42] Erythromycin is a motilin receptor agonist that stimulates contraction of antral and duodenal smooth muscles.[43] In the horse, erythromycin results in a dose-dependent increase in ileocecal emptying.[44] Motility-enhancing effects also have been observed in human patients treated with amoxicillin.[42]

DISEASE CAUSED BY COLONIZATION OF COMMENSAL FLORA

Occurrence of infectious lower respiratory disease in the adult athlete is an example of the conversion of several commensal bacteria into pathogenic organisms because of contamination of a normally sterile site. Changes in upper respiratory mucosal flora and transportation are two important elements that contribute to the development of pleuropneumonia in horses. The tonsillar mucosa of the oropharynx is colonized heavily with *Streptococcus equi,* and necrosis of this tissue occurring during viral infection is associated with spread to the lower respiratory tract.[6] The association of transport as a risk factor for the development of pleuropneumonia in horses was demonstrated in a retrospective study where transport of greater than 500 miles was associated highly with disease.[45] Elevation of the head for an extended duration is an important initiating event. Natural feeding behavior of horses, which results in a lowered head for several hours, is likely an important method for tracheal clearance. Under experimental conditions, elevation of the head for prolonged periods results in an increase in types and multiplication of oral/pharyngeal commensal bacteria within the trachea.[46,47] *Pasteurella, Actinobacillus,* and *Streptococcu*s spp. are the most frequent and prolific colonizers of the trachea after prolonged head elevation. In addition to prolonged head elevation imposed by transport, decreased phagocytosis of equine peripheral neutrophils occurs in horses exposed to extended travel.[47] As a result, common commensal bacteria of the oropharynx and upper airways become opportunistic pathogens.

NOSOCOMIAL INFECTIONS

Nosocomial infections are defined by the Centers for Disease Control and Prevention as "an adverse reaction to toxin or infection that was not present or incubating at the time of admission to a hospital."[48] The Centers for Disease Control also have case definitions for each body site and particular infection in human nosocomial disease. No central mechanism exists for detecting and documenting nosocomial infection in veterinary medicine, and frequently many such infections go unrecognized. Nosocomial infections are becoming a major problem for large animal veterinary teaching and private referral hospitals. Infections with *Serratia marcescens, Acinetobacter baumannii, Staphylococcus aureus,* methicillin-resistant *Staphylococcus* spp., *Enterococcus* spp., and various *Salmonella enteritidis* serovars have been reported in association with nosocomial infection in equine patients.[49-59] Surgical incision infection, joint sepsis, catheter phlebitis, wounds, and diarrhea represent the common clinical nosocomial syndromes reported in horses.[49,51-59] When nosocomial infection involves the acquisition of isolates from the hospital environment, these isolates are more difficult to treat because they frequently undergo high-level antibiotic pressure and attain multiresistance. These organisms also have been shown to cross species lines. Reports of nosocomially transmitted salmonellosis in equine hospital wards are increasing, and outbreaks of *S. enteritidis* serotypes Krefeld, Saint Paul, DT104, and Anatum have demonstrated attainment of

multiple antibiotic resistance over the course of the outbreak.[50-54] Only one study of a nosocomially transmitted *S. enteritidis* (serotype Heidelberg) did not demonstrate significant acquisition of multiple antibiotic resistance over time.[60]

Pathogenesis of Bacterial Infections

The ability of bacteria to gain entry and cause disease results from a combination of factors possessed by the agent itself, environmental conditions, and status of host defenses. Bacteria gain entry through a body surface by direct inoculation or colonize and damage a dermal or mucosal barrier to cause disease. Environmental or risk factors specific for individual diseases increase the probability of successful penetration or colonization and are discussed for specific diseases in various chapters. Innate and specific immunity, which alter host susceptibility to disease, also are discussed elsewhere in this text. Mechanisms that are specific to bacteria and enhance disease are virulence factors. Virulence factors may allow bacteria an advantage to gain entry and disseminate or may cause damage to the host directly once entry has been gained.

Factors That Enhance Entry of Bacteria

ADHESION AND ENTRY

Fibrillar adhesins, nonfibrillar adhesins, and membrane ruffling are virulence factors for bacterial invasion because they aid in colonization of host surfaces. The most common type of adhesin found in gram-positive and gram-negative bacteria are lectins.[61-63] These proteins are highly conserved in bacteria and are important targets for immunoprophylaxis. Although attachment is thought to be the primary role of these proteins, attachment itself results in an intracellular change including actin rearrangements, cell signaling regulation, or actual secretion of bacterial substances into the host cell.

FIBRILLAR ADHESINS

Multiple types of fimbria occur in gram-positive and gram-negative bacteria, with gram-negative papillae the most well characterized.[64] Fimbriae are filamentous appendages that are not flagella and do not function in bacterial conjugation.[62,65] Pili or fimbriae are rod-shaped structures composed of an orderly array of a single protein usually arranged helically to form a cylinder. The tip of the fimbria mediates attachment to carbohydrate moieties on cell surfaces and is integral to bacterial invasion and colonization. Bacteria also can contain multiple types of pili. The bacterial pili themselves and the cellular pathways bacteria use for secretion and formation of pili are targets for pharmacologic intervention.[62] Type I fimbria are distributed in the periplasmic space and translocated to the cell surface by a chaperone/usher pathway. These pili are made up of multiple major pilin subunits compiling a rigid shaft with minor pili proteins composing the flexible tip. The chaperone is a special bacterial cell protein that prevents the pilus from achieving its final figuration while in the periplasmic space located between the inner and outer membrane before assuming its position on the outer membrane. *Salmonella* bacteria and uropathogenic *Escherichia coli* commonly possess type I pili. The K88 and K99 fimbriae of enteropathogenic (EPEC) *E. coli* are also type I pili. *Haemophilus* spp. and *Klebsiella* spp. contain type I pili. Type IV pili are located at the pole of the cell and assembled via the type II secretion system that is called the general assembly pathway.[63,65] These pili are flexible fibers of variable length that can aggregate, are important in the formation of microcolonies, and are responsible for the twitching motion of bacteria. The proteins are secreted into the periplasmic space but remain anchored to the inner membrane. The protein then is assembled and passed through a pore formed in the outer membrane. This type of pilus commonly is found on *Pseudomonas aeruginosa,* enterotoxigenic *E. coli,* and EPEC. Curli fimbriae are solid surface structures that use the extracellular nucleation/precipitation pathway.[63] This pilus is secreted as a soluble protein extracellularly with a second protein, the nucleator, for stability.

In gastrointestinal and urinary tract infections, fimbriae are likely one of the most important virulence factors for successful invasion.[64] Although the importance of *E. coli* adhesive fimbriae is questionable in equine disease, they nonetheless provide a model by which these organelles assist bacteria. Once receptor-mediated attachment occurs, intracellular calcium concentration increases in the host cell.[64] Proteins and protein kinases involved in the breakdown of actin are activated, resulting in the disruption of microvilli. A change occurs in the cytoskeleton of the cells and permeability to ions and water. Ions are secreted, resulting in the classic secretory diarrhea. The specificity of the receptor determines the host species susceptibility to the bacteria itself and the ability of the bacteria to colonize a specific body site or surface.

The classic example of pili-mediated attachment is EPEC; however, receptors for EPEC have not been identified in the horse. Enterotoxigenic *E. coli* have been isolated from foals.[66] Although this bacterium is able to cause gastritis and enteritis in experimental infection, adhesion to foal intestinal mucosa was not demonstrated despite the fact that this same organism could adhere and colonize swine epithelium. The antiphagocytic M-protein of *Streptococcus* spp. is actually a fibrillar protein and also assists, but is not essential for, adhesion. In anaerobic bacteria, antibodies to flagellar proteins FliC and FliD can block *Clostridium difficile* adhesion. These

proteins are thought to mediate intestinal adherence and colonization.[67]

AFIMBRIAL ADHESINS

Afimbrial adhesins are cell proteins that enhance the binding of bacteria to host cells. They also are called "microbial surface components recognizing adhesive matrix molecules."[68-70] Gram-positive organisms possess afimbrial proteins on their surfaces that presumably aid in binding to host cells. The three most commonly studied afimbrial adhesins are those that bind salivary glycoprotein, bind fibronectin, or are composed of lipoteichoic acid.[70,71] Salivary-binding proteins commonly are found in pathogens and commensals of the oral cavity. *Streptococcus* spp. and *Actinomyces* spp. possess these proteins. Fibronectin-binding protein (FBP) is necessary for *Staphylococcus aureus* invasion and binds fibronectin and collagen to form a bridge between the FBP and the host cell integrin (fibronectin integrin $\alpha_5\beta_1$).[72,73] FBPs are essential for invasion of epithelial and endothelial cells as demonstrated by the fact that *Streptococcus pyogenes* FBP mutants do not bind to epithelial cells in vitro.[68-70] Heterologues of FBP have been demonstrated in *S. pneumoniae* of human beings, *S. equi equi,* and *S. equi zooepidemicus.* Other potential equine pathogens that have FDPs on their surface include *Actinomyces* spp., *Enterococcus faecalis,* and *Listeria monocytogenes.*[71,74] Lipoteichoic acid is a common binding factor found in *Streptococcus* group A bacteria, and antibodies to this protein inhibit adhesion of the bacteria to cells.[75] This protein is also important in stimulation of cytokine secretion from the cells during infection, and lipoteichoic acid has been demonstrated in group B *Streptococcus,* including *S. equi equi.*[76] A less commonly described afibrillar adhesin is composed of surface polypeptide chains in *Corynebacterium* that bind to lectin.[77] Binding of these proteins can be abolished by trypsin treatment. Afibrillar adhesins are also present in gram-negative organisms, the most commonly studied are conserved high-molecular-weight adhesion proteins of *Haemophilus influenzae* and *Bordetella pertussis.*[75]

CYTOSKELETAL CHANGES

Binding of bacteria frequently results in cytoskeletal changes within the host cell to enhance susceptibility to invasion. Membrane ruffling is a virulence factor that results in internalization or breakdown of intracellular components to allow for invasion of tissues. The two models of membrane transformation commonly are referred to as zipperlike or triggerlike, and bacteria commonly studied in this phenomenon include *Yersinia* spp., *Listeria monocytogenes, Salmonella* spp., and *Shigella flexneri.* Binding results in actin rearrangement and engulfment of these intracellular bacteria by phagocytic and nonphagocytic cells. *Yersinia* and *Listeria* form a zipperlike relationship in which the tightly adhered bacteria result in actin polymerization and formation of a phagocytic cup.[78] This three-stage process is controlled by different proteins, the first of which initiates actin nucleation and branching of filaments. A second protein stimulates actin polymerization and depolymerization, which is followed by activation of a third protein that ends the process. *Salmonella* and *Shigella* bacteria adhere and secrete proteins that are translocated into the host cell cytoplasm and trigger actin polymerization. Bacterial proteins are secreted into the cell,[79] and an actin-binding protein forms a complex with the cell protein, T-plastin. In the presence of F-actin, an increase in actin bundling of T-plastin occurs primarily in the vicinity of bacterial-host cell contact, resulting in enhanced *Salmonella* invasion. In addition to membrane ruffling, *Mycobacterium avium* and *Salmonella* spp. rely on activation of intracellular guanosine triphosphatases leading to phagocytosis.[80,81]

Factors That Enhance Spread of Bacteria

Once colonization occurs, multiplication and spread of bacteria is enhanced through virulence factors. These factors assist bacteria in evasion of immune defenses, use of the host environment, and breakdown of tissue barriers. Many of these defenses overlap, but the end result is avoidance of destruction by the host and sublimation of host tissues into a new bacterial niche.

EVASION OF IMMUNE DEFENSES

Capsule

One of the most common and potent strategies for avoidance of phagocytosis is the presence of a capsule. The polysaccharide components of capsule are important targets for control of bacterial infections. Immunization strategies against capsular components, such as with vaccination against *Haemophilus influenzae* infection in human beings,[82,83] has been successful in the control or elimination of this disease.

Bacteria are diverse and many have a capsule, but assembly and structure of the capsule is remarkably similar between bacteria. The *Escherichia coli* capsule is representative of the majority of gram-negative capsule types.[84] Three genetic regions control the development of capsule, and all are targets for intervention. The first region controls transport and assembly of the mature polysaccharide through the outer membrane. The second genetically encoded region controls formation and polymerization of polysaccharide itself. The complexity of the resulting protein product depends on the size of this

region. The third genetic locus contains genes that are involved in translocation of the polysaccharide through the inner membrane.

The capsule of bacteria is composed mainly of polysaccharide, which is inherently a poor immunogen. Ultrastucturally, the capsule in *E. coli* and *Klebsiella pneumoniae* is a fine fibrillar meshwork covering the bacteria surface; however, the capsule of *Klebsiella* is approximately 20 times thicker than that of *E. coli*.[85] In Enterobacteriaceae, the K antigen is one of the major antigens of this complex and expression is temperature dependent.[86] The presence of certain K antigens and a large capsule, as studied in *Klebsiella* virulence, is associated highly with increased pathogenicity and resistance to phagocytosis.[87,88] The other major antigen is the lipopolysaccharide without the lipid A component of the molecule. A third minor component, colanic acid, appears to exhibit antiphagocytic activity in some *E. coli.* Classically, the presence of capsules on *E. coli* have been shown to prevent complement-mediated phagocytosis.[89] Lipopolysaccharide itself activates complement, and the capsule prevents immune activation by concealing the lipopolysaccharide molecule. Introduction of the genes associated with production of a more viscous capsule into a bacteria change an avirulent phenotype into a virulent phenotype.[90]

As early as 1928 the engulfment and digestion of *Streptococcus pneumoniae* was observed to be associated with lack of capsule.[91] By 1940 this virulence factor was understood to be genetically encoded.[92] Early studies with *S. equi equi* demonstrated that resistance to phagocytosis was associated with an increase in capsule and M protein,[93] and in a model of *S. equi zooepidemicus* infection in mice, enhancement of virulence was associated with increased capsule and resistance to phagocytosis.[94] Although colonization of the guttural pouch occurs with nonencapsulated *S. equi equi* strains, induction of lymphadenopathy is correlated more to the capsular strains. Recent studies have shown that when M protein content is kept constant, the amount of capsule actually is correlated with resistance to phagocytosis.[95] Resistance to in vitro phagocytosis can be abolished with treatment with hyaluronidase. In support of this, phagocytosis of *S. equi equi* and *S. equi zooepidemicus* can be enhanced significantly in a murine model by induction of specific immunity against the hyaluronan component of this capsule.[96]

The two most important underlying components of abscess formation are (1) resistance to phagocytosis and (2) failure of bacterial clearance. Capsules of anaerobic bacteria are unique, and their role in virulence may account directly for the formation of abscesses within the host. The capsule of *Bacteroides fragilis* has two distinct polysaccharides composed of repeating subunits with oppositely charged groups (zwitterion).[97] This polysaccharide complex injected alone promotes the induction of abscess. Development of antibody against these polysaccharides prevents abscess formation.

Infection of rodents with the encapsulated form of *Bacteroides* spp. and *Fusobacterium* spp. results in the formation of intraperitoneal abscesses, whereas nonencapsulated bacteria do not cause abscessation.[98-100] Higher mortality is associated with encapsulation in these models. Synergism of capsular anaerobes with other bacteria occurs; nonencapsulated bacteria have enhanced survival in abscesses and produce capsules when cultured or inoculated with encapsulated bacteria.[99]

Anticomplement Factors

Similar and overlapping with the function of capsules are structural proteins that block complement. The O side chain of lipopolysaccharide on gram-negative bacteria is an anticomplement factor.[101] The longer the side chain, the farther the distance between phagocytes and the bacteria. Many capsules contain sialic acid, the interaction of which with O antigen prevents the formation of C3 convertase.[102] Bacterial enzymes are formed by *Streptococcus* spp. and other organisms that damage the polymorphonuclear cell chemoattractant C5a.[103-106] Production of a protein in *Salmonella* spp., encoded by the *rck* gene, prevents insertion of the C9 fragment of complement into the bacterial membrane.[9]

The M proteins of *Streptococcus* spp. are considered important for resistance to phagocytosis. Specifically, for *S. equi equi,* the M protein appears to decrease deposition of complement on the surface of the bacteria. The hypervariable region of the M protein of *S. pyogenes* and *S. pneumoniae*[107,108] confers this resistance to phagocytosis.[109] The M protein of *S. equi zooepidemicus* is 90% homologous in the hypervariable region and likely mediates the same function.[93] This mechanism for complement resistance appears to be through enhancement of binding of fibrinogen to the bacteria in the presence of M protein.[110-112] When fibrinogen is present on the outer surface of the bacteria, phagocytosis is blocked.

Phagolysosomal Survival

The intracellular environment should be inhospitable for bacteria, yet many organisms are ingested by phagocytic cells and use the intracellular environment to multiply and disseminate. In normal phagolysosomal fusion the phagocytic vesicle first becomes fused with a host cell endosome. Shortly thereafter, fusion with the lysosome occurs. Several digestive proteins are released within the lysosome and a drop in pH occurs, resulting in inactivation and digestion of a foreign protein or microorganism. *Shigella* spp. and *Listeria monocytogenes* are bacteria that escape

the phagocytic vesicle to multiply in the host cell cytoplasm.[113]

Extended survival (and replication) in the phagosome itself is an important strategy for dissemination of bacteria. The two main mechanisms for phagosome survival include (1) inhibition of phagolysosomal fusion and (2) blockage of the formation of lysosomal enzymes once phagocytosis occurs. Before escape from the phagosome, *Listeria* modulates maturation of the phagosome by delaying fusion with the lysosome.[114] One of the earliest observations of *Mycobacterium tuberculosis* infection in cell culture was uninhibited multiplication within macrophages.[115] Later studies determined that *Mycobacterium* and *Legionella* cause a change in the maturation of the phagosome.[116] Once the phagosome is infected, many lysosomal proteins appear in it, yet others do not, indicating a manipulation of the environment after fusion. Engulfment of *Salmonella* in the phagosome appears to induce formation of an actual phagolysosome, but the organism survives nonetheless.[117] Acidification occurs, but production of reactive oxygen species is decreased. In addition, *Salmonella* appears resistant to reactive oxygen, which is controlled by a number of *oxy* genes that are upregulated in response to oxygen stress. This occurs rapidly, within 20 minutes of intracellular uptake of the bacteria.[118] Many bacteria that are resistant to reactive oxygen are able to make catalase and superoxide dismutase.

Inhibition of phagolysosomal fusion itself is an important protective mechanism of bacteria. *Rickettsia, Neorickettsia* (formerly *Ehrlichia*), and *Rhodococcus* spp. bacteria appear to inhibit phagolysosomal fusion. More detail regarding mechanism of this phenomenon is given in the section on equine *Ehrlichia*. *Rhodococcus equi* also inhibits phagolysosomal fusion, and although the mechanism of inhibition is unknown, opsonization by *R. equi*–specific antibody results in enhanced fusion and killing.[119,120] Resistance to phagolysosomal fusion appears to depend on the presence of the 90-kd virulence plasmid.[121]

ADAPTATION TO HOST ENVIRONMENT

pH Resistance

The acid tolerance response is important primarily for intracellular bacteria as a way to survive the acidification within a mature phagolysosome. Many bacteria including *Listeria monocytogenes, R. equi,* and *Salmonella* spp. are able to withstand highly acidic environments.[120,121] Genes that control virulence in *Salmonella* actually are upregulated by an acidic environment.

Bacterial Nutrition

Nutrition of bacteria is associated intimately with cellular and tissue environments. Although bacteria use iron for many enzyme systems, the relationship between bacteria and iron is more complex. The proper level of iron is important because iron is required for the production of reactive oxygen intermediates. The *fur* gene in *Escherichia coli* was identified first as the major regulator of iron acquisition.[122] Homologues of this gene have been identified in many other bacteria, including *Salmonella* spp., *Vibrio* spp., *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Bacteroides fragilis*.[122-127] This gene regulates the amount of iron handled by the bacterial cell depending on the host stores. In times of iron stress, the gene is downregulated to allow for more iron uptake from the environment by the bacterial cell. In addition, bacteria possess siderophores, which are potent chelators of iron.[128] These siderophores are secreted outside of the bacterial cell, where they take up iron, and are taken back into the cells through a receptor-mediated process. After internalization, iron is cleaved and used by the bacteria. Resistance to carbon and nitrogen starvation are two other means assisting bacterial survival in inhospitable environments.

DAMAGE TO HOST TISSUES

Toxins

Exotoxins are virulence factors secreted by bacteria generally to aid spread of infection. Gram-positive and gram-negative organisms secrete an array of exotoxins. Classically, three types of exotoxins occur[129]: (1) the A-B form with two distinct parts for binding and for enzymatic action, (2) toxins that form pores within host membranes, and (3) superantigens that form a bond between the major histocompatibility complex class II receptor of macrophages, resulting in release of various T helper cell–mediated cytokine cascades. Several examples of common equine pathogens follow.

Toxins of *Staphylococcus aureus* are well characterized and are associated directly with the pathogenesis of disease.[130] Toxins that may be important for disease in the horse include the four hemolysins, toxic shock syndrome toxin 1 (TSST-1), exfoliative toxins, and leukocidin. Few overt syndromes are recognized in the horse that are caused specifically by *S. aureus*. However, *S. aureus* is the most common bacteria isolated from cellulitis and joint infections in adult horses. Cellulitis induced by *S. aureus* in the horse may be similar to wound-associated toxic shock in human beings. Toxic shock syndrome is a disease of increased capillary permeability characterized by hypotension, hypoalbuminemia, and edema. The toxins of *S. aureus* work in concert to (1) induce massive release of cytokines, (2) increase sensitivity to cytokines, and (3) damage endothelial cells directly. The superantigen toxin of *S. aureus* forms a bridge between the major histocompatibility complex receptor on macrophages and the T cell receptor resulting in a massive release of interleukin-2 (IL-2). The downstream effect is T cell

proliferation and induction of type 1 cytokines that stimulate release of proinflammatory cytokines from macrophages. Exfoliative toxin also induces T cell proliferation. TSST-1 and exfoliative toxin of *S. aureus* enhance the effects of endotoxin in cell culture and live animal experiments. In rabbits the lethal dose of endotoxin is decreased by 100-fold. The TSST-1 binds directly to endothelial cells and in endothelial cell culture demonstrates cytotoxicity. A dose-dependent induction of leakage across monolayers also occurs. Exfoliative toxin and TSST-1 have been identified in equine *S. aureus* isolates associated with severe phlegmon and metritis.[131,132]

Alpha-toxin has been studied intensely and is directly toxic to many cell types.[129] This toxin can damage erythrocytes, skin, and nerve cells and is lethal.[130] At high concentrations, molecules of the toxin polymerize and form pores in monolayers of cells resulting in permeability of the cells. The arachadonic acid cascade is activated, resulting in thromboxane- and prostacyclin-mediated vasoconstriction in the face of edema formation. Alpha-toxin also appears to act directly on platelets, resulting in release of procoagulant factors. Increased permeability and swelling of cells may be important in the pathogenesis of dermal and neural damage. Myelin sheath destruction is the lesion observed in rabbits. The toxin, β-hemolysin or sphingomyelinase C, is produced in high concentrations in many *S. aureus* isolates, and the degree of pathogenicity to erythrocytes likely is determined by sphingomyelin content of erythrocytes of different strains and species. This toxin has high amounts of phosphorylase C activity in the presence of Mg^{2+}. The presence of this toxin appears to be correlated with enhanced growth characteristics. The toxins γ-hemolysin and leukocidin appear to cause degranulation of leukocytes, with leukocidin being the more potent of the two. Finally δ-toxin is toxic to erythrocytes and cellular organisms and also is lethal at high doses.

Clostridial Infections

Clostridrium spp. mediate clinical disease by producing exotoxins. The clostridial bacteria causing botulism and tetanus excrete neurotoxins to which horses are highly susceptible. The main toxins of botulism and tetanus are remarkably similar even though they exert effects that appear to be opposites, with botulism causing a flaccid paralysis and tetanus causing a spastic paralysis.[133] Both toxins share amino acid sequences of metalloproteinases that are most similar to zinc-requiring endopeptidases. With botulism, at least six serologically different neurotoxins occur, with most reports in horses caused by type B toxin, although A, C, and D also have been identified.[133-137] Only one tetanus neurotoxin is responsible for the clinical signs of spastic paralysis.

Neurotoxin is secreted as a progenitor and must be cleaved by proteases (trypsin) to the derivative toxin to produce clinical signs. The active toxin consists of a light and heavy chain (A-B type toxin) that mediates paralysis by blocking acetylcholinesterase in a three-step process.[129,138] The first step is rapid and involves recognition and binding of the neurotoxin to its receptor on the nerve. In the second step, translocation occurs as the active site of the molecule is internalized in the nerve cell ending by endocytosis. The acidified vesicle induces a conformational change in the toxin so that it can be translocated to the cytosol. The third and final step is the slow step in which cell neuroproteins called synaptopeptidases are cleaved, preventing acetylcholine release. The proteins that are cleaved by the neurotoxin include synaptobrevin, synaptosome-associated membrane proteins, and syntaxin. The protein synaptobrevin is a component of the neurotransmitter cell membrane, is essential for neurotransmitter release, and is involved in exocytosis of the neurotransmitter vesicle and axonal growth. Syntaxin is important for vesicle and cell membrane fusion. The inactivation of one or more of these proteins accounts for the prevention of release of synaptic vesicles. The different botulinum neurotoxins exhibit variable specificity to these proteins. Types B, D, F, and G are active against synaptosome-associated membrane proteins, whereas A, C, and E are active against synaptobrevin, and C is the only form that cleaves syntaxin.

The difference in clinical signs noted between *C. tetani* and *C. botulinum* is a reflection of the binding and level of activity of the respective toxin. *C. botulinum* binds to peripheral nerves, and *C. tetani* binds to cells within the central nervous system. Furthermore, *C. botulinum* toxin binding results in prevention of downstream release of other neurotransmitters, such as γ-aminobutyric acid. Injection of *C. tetani* toxin intravenously in low doses results in flaccid paralysis.

The main pathologic features of disease cause by *C. perfringens* are edema, necrosis, and death, regardless of the site of action.[138] Toxins elaborated by these clostridia include lethal and nonlethal toxins that cause necrosis and hemolysis and frequently contain lecithinases and lipases. *C. perfringens* has four major lethal toxins, an enterotoxin, and three minor toxins. Recent genetic evaluation of equine isolates associated with diarrhea indicate the majority of isolates are type A, the toxic affects of which are mediated by α-toxin.[139] The *cpe*-toxin or enterotoxin has been detected by enzyme-linked immunosorbent assay in 16% to 19% of equine isolates, so disease has been linked to enterotoxigenic type A and nonenterotoxigenic type A strains.[18,140] Individual case reports exist of neonatal diarrhea associated with type B (β- and ε-toxins), type C (β-toxin), and type D (ε-toxin) strains, which also carry the α-toxin.[141,142]

Classic experiments demonstrate that injection of purified α-toxin into mice results in hemolysis and death, and recent evidence confirms the role of α-toxin as the major virulence factor of gas gangrene.[138] α-Toxin is one of several zinc metalloproteinase enzymes of bacteria including that of *Listeria monocytogenes, Bacillus cereus,* and other *Clostridium* spp. One enzyme is phospholipase C, which is able to hydrolyze phosphatidylcholine (lecithin) and sphingomyelin.[143,144] This toxin is responsible for cardiovascular collapse and death, likely by increasing vascular permeability. At the cellular level, this toxin can cause platelet aggregation and hemolysis and is cytotoxic and myotoxic. Vascular activity is mediated by upregulation of adhesion molecules in leukocytes and endothelial cells. Endothelial cells and macrophages produce platelet-activating factor in response to toxin exposure. Muscle fibers are damaged by the formation of intravascular aggregates within the muscle.[143]

Recent work with β-toxin and ε-toxin indicates that these toxins act as pore formers.[145,146] Early research into the mechanism of action of β-toxin indicated a role for catecholamine release because of the sudden increase in heart rate and drop in blood pressure when the toxin was injected into experimental subjects.[138] β-Toxin polymerizes and forms pores that act as selective ion channels, the generation of which within cell membranes effectively creates a neurotoxin.[145] Work with ε-toxin indicates that this toxin is able to form large pores within membranes, resulting in a flux of potassium outward and chloride and sodium inward. Later ion flux is dominated by movement of calcium into the cell.[146] This flux results in irreversible morphologic changes in the cell membrane and death of the cell.

Enterotoxin binds directly on the cell membrane, causing a change in ion movements that affects cellular metabolism.[138] Similar to the actions of these other toxins, most of the damage is mediated by increased calcium levels within the cell, affecting cell membrane function and permeability. The newly recognized β_2-toxin has been associated with intestinal disorders in horses; however, the exact mechanisms of action of this toxin are unknown.[147]

C. difficile has five toxins, of which toxins A and B are thought to be most important in development of disease.[148,149] The evidence to date indicates that toxins A and B mediate the pathogenesis of *C. difficile* diarrhea.[149] Research suggests that the formation of the classic pathologic lesion, the pseudomembrane, results from the combined actions of toxin A, toxin B, and IL-8. Classically, toxin A is considered an enterotoxin and lethal, whereas toxin B is a virulent cytotoxin. Toxin A appears to be the primary mediator of fluid accumulation.[150,151] The toxin is thought to bind to an oligosaccharide receptor and is internalized immediately. After a lysosomally mediated acidification step, a rapid fall in intracellular adenosine triphosphate concentration occurs, followed by release of mitochondrial cytochrome *c* and generation of reactive oxygen intermediates. Injection of toxin A results in fluid accumulation, cell necrosis, and recruitment of inflammatory cells. One mechanism of fluid accumulation appears to be by interruption of actin filaments and destruction of tight junctions. Toxin A also results in neutrophil recruitment possibly mediated by the direct action of the toxin on macrophages to stimulate release of IL-1, tumor necrosis factor α, and leukotrienes. In cell culture, toxin A can cause IL-8 secretion (a potent recruiter of neutrophils), cell detachment, and apoptosis of separated cells. Direct activation of macrophages and secretion of IL-8 is through a calcium and calmodulin–dependent mechanism that results in direct nuclear upregulation after nuclear translocation of transcription factors NF-κβ and AP-1.[150] Toxin B is more cytotoxic than toxin A, especially to human epithelial cells.

Cell Death

Apoptosis is a distinctive morphologic process that results in cleavage of nuclear material and scavenging of unwarranted cells without immune activation.[152] Apoptosis or programmed cell death is an important pathway for complex organisms to deal with damaged and diseased tissue. Apoptosis avoids the release of the tissue damaging enzymes and nonspecific elimination of tissue that occurs in cellular necrosis. Several bacteria modulate the host apoptotic pathways to enhance survival.[153] *Shigella flexneri, Salmonella typhimurium,* and toxins of *Staphylococcus aureus, Pseudomonas* spp., and *Corynebacterium diphtheriae* have demonstrated programmed cell death as a consequence of cellular infection or exposure.[152,154] A protein of *Shigella flexneri,* IpaB, induces apoptosis by binding to and activating the cellular enzyme caspace 1, which induces apoptosis of macrophages.[113] *Staphylococcus aureus* α-toxin, presumably escapes the macrophage after engulfment and induces host cell apoptosis.[130] The TSST of *S. aureus* induces B cell apoptosis and blocks immunoglobulin production.

Pathogenesis of Fungal Infections

Of the 250,000 species of fungi, fewer than two hundred are true pathogens.[155] Superficial mycoses affect the hair shaft and the superficial epidermis. Cutaneous mycoses (dermatophytosis) infect the epidermis, dermis, hair, and nails of animals, and *Microsporum, Trichophyton,* and *Epidermophyton* are the most commonly associated pathogenic genera. Subcutaneous tissues can become infected with *Sporothrix, Conidiobolus, Basidiobolus,* and

members of the Dematiaceae fungi, which cause the chromoblastomycosis, mycetoma, and phaeohyphomycosis infections. Most of these infections are introduced by penetration through skin or opportunistic invaders of damaged skin surfaces. *Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis,* and *Paracoccidioides brasiliensis* are the four most important systemic fungal pathogens. The most common opportunistic infections include *Candida albicans, Aspergillus* spp., *Cryptococcus neoformans, Mucor* spp., and *Pneumocystis carinii.*

Factors That Enhance Entry of Fungi

Fungal virulence factors may be more complex than those of bacteria because of the higher degree of opportunism that occurs primarily because of a change in host status. Subtle factors may combine with host status to result in a certain fungus attaining a virulent state. For example, the typical fungal wall is composed of three major polysaccharides: mannose, glucans, and chitin. Chitin mutants of *Candida albicans* are less virulent when tested in rodent models than wild-type fungi.[156] A mutant *C. albicans* that cannot synthesize complex mannose oligosaccharides does not adhere to other yeast and epithelial cells and has lost virulence in a guinea pig model.[157] Both of these mutants can proliferate in vitro normally, and whether this is an actual virulence factor is unclear.

As in bacteria, adherence is an important characteristic for infection and colonization of the host. Adhesins have been identified in *C. albicans* and *B. dermatitidis.* Two genes have been associated with adhesion in *C. albicans.* The first is a glycoprotein that has sequences consistent with agglutinating activity. Transformation of this gene into other nonadherent fungal species results in adhesion of the transformed yeast to cells.[158] *C. albicans* also has integrin-like proteins, the disruption of which results in diminished hyphal growth, adhesion to cells, and loss of virulence in mice.[159,160] The *B. dermatitidis* adhesin mediates binding to human monocyte-macrophages through the CD14 receptor.[161]

Factors That Enhance Spread of Fungi

EVASION OF IMMUNE DEFENSES

Many fungi have polysaccharide capsules that help them resist phagocytosis and immune activation. The capsule of *Cryptococcus neoformans* inhibits leukocyte accumulation, cytokine secretion, and macrophage phagocytosis.[162] Mutants without capsules are highly infective and avirulent. As indicated earlier, many fungi are engulfed by macrophages, and intracellular survival is mediated by virulence factors. Macrophages are capable predators of *Candida albicans,*[163] *H. capsulatum,*[164] and *B. dematitidis. H. capsulatum* is primarily a yeast in vivo, and this form infects macrophages. Phagolysosomal fusion occurs at a normal rate,[165] but blockage of acidification of the phagolysosome occurs.[166]

ADAPTATION TO HOST ENVIRONMENT

Morphology and Temperature

Adaptability to the host environment is also a trait that enhances fungal virulence. Fungal dimorphism, which is the ability to adopt another morphologic state, clearly is tied to virulence. Mutants of *C. albicans* that cannot switch to the hyphal form are avirulent in certain in vivo models, although both forms likely contribute to pathogenesis.[167] In a second adaptation, phenotypic switching, colonies of fungi change during in vitro growth. *Candida albicans, Cryptococcus neoformans,* and *H. capsulatum* display phenotypic switching, and different phenotypes are associated with degrees of virulence.[168-171] *H. capsulatum* spontaneously gives rise to mutants that have less capsule, are less virulent, and are not cytotoxic to macrophages.[170] The signal for dimorphic fungi to change form is usually temperature. Many fungal species germinate within the host with increased temperature, allowing for dissemination within the host. Calcineurin is found in many yeast and mammalian cells and in *C. neoformans.* This protein mediates the ability of the fungus to grow at 37° C.[172] Temperature and heat shock also are mediated by the calcium-dependent protein cyclophilin B in *Aspergillus.*[173] Adaptation to mammalian pH is controlled genetically in *Candida albicans.* Mutants display abnormal cell morphology at physiologic pH ranges.[174]

Nutrients

Nutrient requirements that affect virulence include melanin, iron, and calcium. Melanins are present in the wall of *Cryptococcus neoformans,* and melanin can scavenge reactive oxygen intermediates, making the organism resistant to the oxidative burst of neutrophils.[175] Pathogenic fungi have siderophores and high-affinity ferric iron reductase to acquire iron from low-iron environments.[176,177] *H. capsulatum* secretes a calcium-binding protein that enhances calcium uptake from calcium-poor environments.[178,179] Without this protein, *H. capsulatum* cannot form colonies and does not survive in cultured macrophages.

Damage to Host Tissues

TOXINS

Exposure to pathogenic and saprophytic fungi is an everyday occurrence. Respiratory contamination and infection is important for many species, but skin penetration and

dissemination from necrotic gut are important portals for large animals also. After initial infection, dissemination depends on previous damage to host tissues, deeper mechanical penetration, or actual invasion of new tissues. *Candida albicans* can grow through and replace cell membranes.[163] True molds invade blood vessels and grow along the intima of the vessels. Fungi secrete many degradative enzymes, including proteinases, phosphatases, and DNAses to surmount structural barriers.[180] Aspartyl proteinase genes allow more persistent colonization of host surfaces and deeper penetration.[181]

When *Coccidioides immitis* invades the host, the fungi form endospores. Endospores secrete proteinase and urease, which likely aid in the breakdown of pulmonary tissues.[182-184] The two proteinases of *Aspergillus fumigatus* break down elastin, a major component of lung tissue.[185,186] Phospholipase activity has been demonstrated in *Candida albicans, Cryptococcus neoformans,* and *A. fumigatus.*[187] Strains of *Candida* spp. with high amounts of this enzyme have enhanced virulence,[188] and abolishing this activity results in decreased adherence of the organism.[189] Host eicosanoids enhance fungal colonization. Recent evidence demonstrates production of eicosanoids by dermatophytes and systemic fungi.[190]

APOPTOSIS

Fungi induce apoptosis by a direct effect of a fungal toxin or following host cell cytoskeleton rearrangements.[191] The gliotoxin of *A. fumigatus* can induce DNA fragmentation and apoptosis in macrophages.[192] This toxin also has many other immunosuppressive qualities, including inhibition of the neutrophil respiratory burst and T cell activation.

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2.2—Mechanisms of Establishment and Spread of Viral Infections

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Viral infections are responsible for some of the most medically and economically important diseases of horses. A few notable examples include influenza, equine rhinopneumonitis and abortion (Herpesviridae), African horse sickness (Reoviridae), equine infectious anemia (Retroviridae), various encephalitis viruses (Alphaviridae), and most recently in the United States the West Nile virus (Flaviviridae). Specific therapy of viral infections remains a significant challenge because antiviral drugs

are generally ineffective, impractical, or cost-prohibitive for the treatment of horses. Treatment of most viral infections focuses on supportive care of the affected organ system(s) and control of secondary complications such as bacterial infection. Currently, control of most clinically significant viral diseases in horse populations relies on vaccination, quarantine, or even destruction of infected animals.

Despite the great significance of some viral infections, recognizing that many equine viruses are ubiquitous, weakly pathogenic, or not associated with any known disease under normal circumstances is also important. Some examples include equine adenovirus,[1] respiratory and enteric reoviruses[2] (the term *reo* is derived from the acronym for *r*espiratory *e*nteric *o*rphan, indicating that these isolates have not been associated with disease), and equine herpesvirus (EHV) types 2 and 5.[3] Some host-virus relationships may be mutualistic in that virally derived genetic elements are theorized to benefit the host by facilitating genetic variability and evolution.[4] Thus many viruses are of no practical clinical significance, and no control efforts are warranted. For this reason, one should never assume that the recovery of a virus from a clinical specimen indicates significance without proof that the virus can cause the disease in question.

Veterinary virology is a rapidly changing field. New diseases continue to emerge or be discovered; a dramatic example is the Hendra paramyxovirus that appeared in Australia in 1994, killing horses and human beings.[5] More recently, another paramyxovirus, the Salem virus, has been identified in the United States, although the clinical importance of this virus is unclear.[6] Less dramatic but of more relevance to most equine veterinarians is the association of bovine papillomavirus with sarcoids.[7,8] A number of equine diseases occur in which viral involvement has yet to be excluded, including Theiler's hepatitis[9] and lymphosarcoma.[10] Viral origins also likely will be discovered for diseases in which viruses were not suspected previously to play a role; in human beings and mice, viruses have been proposed to have a role in everything from diabetes to obesity.[11,12] However, the greatest advances in veterinary virology are in understanding the molecular biology of viral replication, virus-cell interactions, and virus-host interactions. Related advances in molecular biology techniques, such as the polymerase chain reaction and immunohistochemistry, also are providing sensitive, specific, and rapid tools for the diagnosis of viral infections. In the realm of antiviral drugs, aggressive searches are ongoing for effective and economical drugs for treatment of human beings, and in the near future, antiviral drugs likely will be a realistic therapeutic option for equine veterinarians.[13,14] Finally, significant advances in the design of vaccines likely will improve the efficacy of immunization.

This chapter outlines the general ways that viruses cause disease and describes the most important virus-cell and virus-host interactions that result in pathologic conditions. Although the focus is on equine viruses where possible, the principles described are generally not species dependent, and no attempt is made to limit the discussion to recognized equine viral pathogens or diseases. Discussion of a particular mechanism or virus also should not be taken to suggest that this mechanism or type of virus has been documented in horses. Where possible, supporting references have been selected to include review articles or texts for additional information about key concepts.

Viruses and Virus-Cell Interactions

An in-depth discussion of viral structure, taxonomy, and replication is beyond the scope of this chapter, and the reader is referred to textbooks of veterinary or human virology for more detailed information.[15,16] However, a brief overview is presented to emphasize those features that have clinical relevance.

VIRUS STRUCTURE, TAXONOMY, AND REPLICATION

The fundamental structure of all viruses is a DNA or RNA genome enclosed by a coat of protein called the capsid (Figure 2.2-1). For viruses that are enveloped, the capsid is enclosed further by a host cell–derived lipid membrane into which viral proteins have been incorporated. In addition to protecting the viral genome, the capsid and other associated structural proteins (e.g., matrix proteins) are important for virus assembly, packaging the viral genome, releasing the genome into a target cell, and for non-enveloped viruses, providing receptors that bind to host cells. For enveloped viruses the receptors are incorporated into the lipid membrane. The primary clinical significance of these features is that enveloped viruses, because of their fragile lipid membrane, are highly susceptible to inactivation by heat, desiccation, or detergents, and transmission typically requires direct exchange of body fluids, short distance aerosols, or arthropod vectors. In contrast, nonenveloped viruses (e.g., equine rotavirus) are resistant to physical inactivation, and environmental contamination is more likely to be a significant factor in their transmission.

The composition of the viral genome is an important basis for virus classification (Figure 2.2-2). The type of viral genome also determines the strategies required to replicate the genome and transcribe messenger RNA (mRNA) (Figure 2.2-3). Viral genomes may be single-stranded RNA, double-stranded RNA, single-stranded DNA, or double-stranded DNA. The genomes of single-stranded RNA viruses may be of "positive" polarity

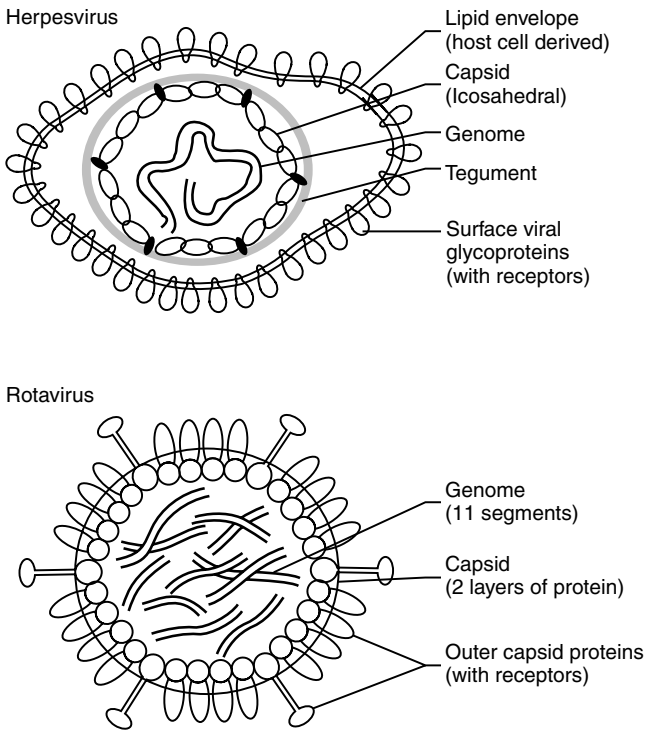

Figure 2.2-1 Schematic representations of basic viral structure. The basic structure of an enveloped virus is shown by the drawing of a herpesvirus. The basic structure of a non-enveloped virus is shown by the drawing of a rotavirus.

in which the genome also serves directly as mRNA for translation of viral proteins. To replicate genomes, these viruses must first synthesize a strand of complementary RNA that can be used as a template to replicate genomes and transcribe new mRNA. Retroviruses are a subset of single-stranded, positive polarity RNA viruses that use their RNA genome as template to produce double-stranded DNA, which in turn is used for the transcription of mRNA and new viral genomes. Single-stranded RNA viruses may also be of "negative" polarity in which the genome is antisense to mRNA, and synthesis of a complementary RNA strand is required to serve as mRNA and as template for new genomes. The clinical significance of these types of replication strategies is that they require polymerases and other enzymes not normally found in eukaryotic host cells. Eukaryotic cells, which exclusively use DNA as genetic material and as templates for mRNA transcription, use DNA-dependent DNA polymerases and DNA-dependent RNA polymerases for these functions, respectively. RNA viruses require RNA-dependent RNA polymerases to replicate or transcribe RNA, or RNA-dependent DNA polymerase (reverse transcriptase) to produce DNA from an RNA template. These unique viral enzymes are important targets for antiviral drugs because they can be used selectively to inhibit viral replication. The most common antiviral drugs currently available are nucleotide analogs that are used selectively by viral but not cellular enzymes and result in defective DNA or RNA.[17,18] Although most DNA viruses follow the eukaryotic pattern of replication and transcription, in many cases these viruses produce homologs of host cell enzymes that are sufficiently unique to make them selectively susceptible to nucleotide analogs. Additional targets for current and future antiviral agents are viral proteins used for translation and posttranslational protein processing.[17]

Viral polymerases and the genetic organization of viruses are also important for rapid antigenic variation and immunologic evasion. Viral RNA polymerases are low fidelity and lack proofreading functions and thus randomly will introduce errors into new RNA at an average rate of about one nucleotide mismatch per 10,000 bases copied.[19] Therefore in a population of viruses, virtually every individual virus differs slightly, and this population is referred to as a quasi-species. Although many of these mutations are neutral or even deleterious, in the face of selective pressures such as the host immune responses or antiviral drugs, this genetic plasticity allows rapid development of resistant virus populations.[20] Secondary structures or certain sequences in the viral genome may facilitate polymerase errors further at selected regions that are important for immune evasion, such as in sequences that code for neutralizing epitopes.[19] The genomes of some viruses, such as influenza, are comprised of separate segments, which allows reassortment of entire gene segments and sudden and dramatic changes in antigenicity.[21]

VIRUS LIFE CYCLE

All viruses are obligate intracellular parasites. Viral replication can occur only within living cells, and all viruses to some extent depend on the host cell synthetic machinery. The life cycle of all viruses includes the following steps: attachment to the target cell, entry into the cell, uncoating and release of the viral genome, transcription and translation of viral proteins, replication of the viral genome, assembly of new virions, and release of progeny virions[15,16] (Figure 2.2-4). Although the biochemistry of these steps is beyond the scope of this discussion, recognizing that they are all specific, energy-requiring interactions between the virus and the host cell is important. The inability of the virus to interact appropriately with a cell at any of these steps prevents replication in that cell type and defines the tropism of the virus. Any of these steps are also important potential targets for antiviral drugs and host immune responses.

One of the most critical virus-cell interactions is attachment and entry. This initial step is one of the most important determinants of species susceptibility, host cell tropism, and is an important target for antiviral antibodies that neutralize infectivity. Attachment and entry requires

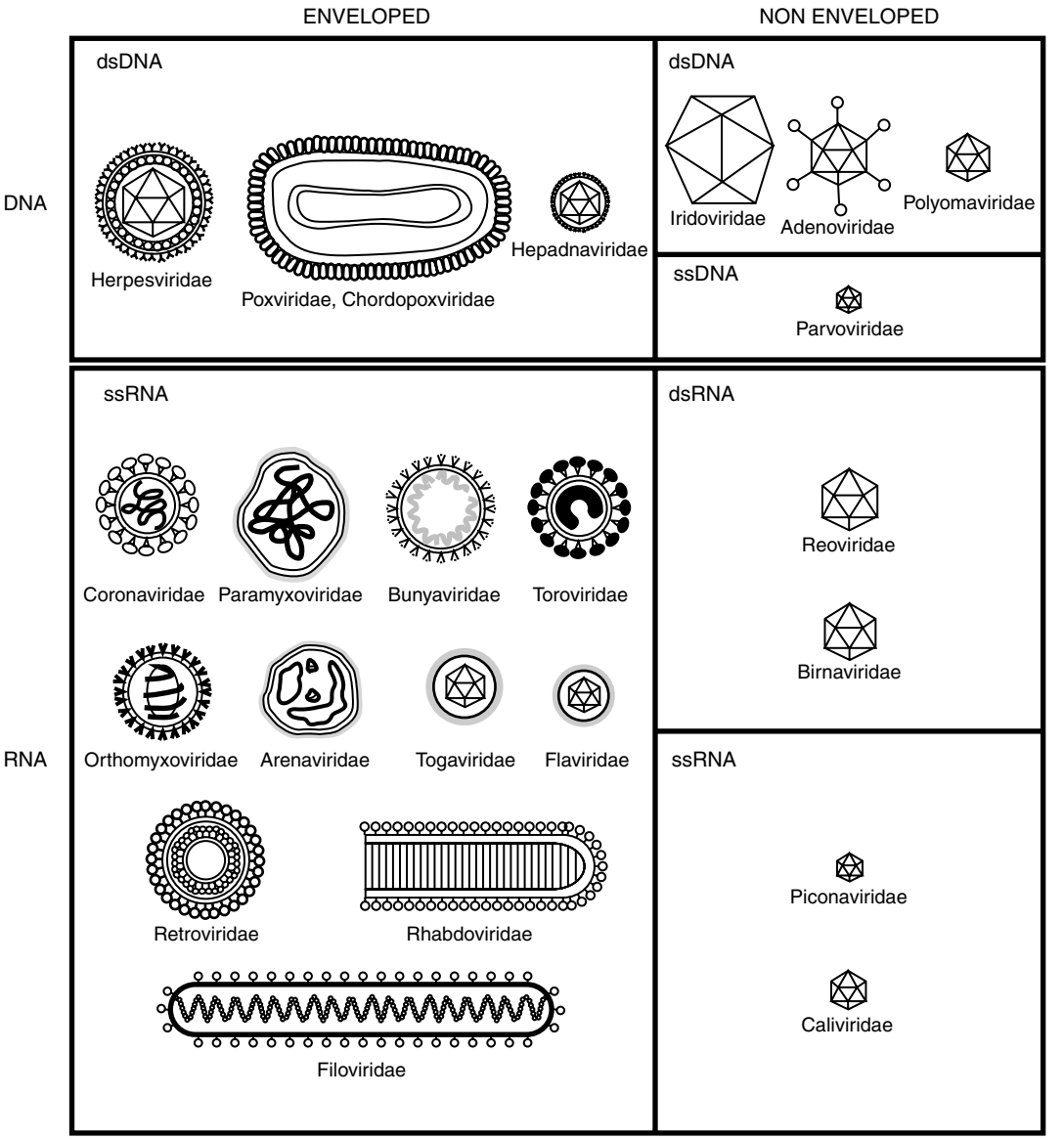

Figure 2.2-2 Virus family classification based on genome composition and presence of envelope or absence of envelope. The relative size of the viruses to each other is also shown.

a specific interaction between a viral receptor and a cell-surface protein that acts as the host cell receptor. Many viruses also require interaction with additional cell surface molecules (co-receptors) for successful attachment and entry. Influenza provides a good example of this process. The influenza virus hemagglutinin molecule binds to sialic acid residues of cell-surface glycolipids or glycoproteins. Binding induces a conformational change in the hemagglutinin protein, exposing a cleavage site to a host cell protease. Cleavage of hemagglutinin induces fusion of the viral envelope to the host cell membrane and release of the capsid into the cytosol.[22,23] Equine influenza does not infect human beings because the hemagglutinin molecules do not recognize human sialic acid molecules.[24] Within the horse, equine influenza infection is restricted to respiratory epithelial cells by the distribution of the appropriate host cell protease.[22] Viruses may infect other cell types by using a different host cell receptor for attachment and entry. Highly pathogenic strains of avian influenza are able to spread systemically because of mutations in the hemagglutinin that allow cleavage by host cell proteases found on cells outside of the respiratory tract.[22] Other ways in which a virus can infect multiple cell types are using a host cell receptor that is present on different cell types or infecting a single cell type that is present in different tissues (e.g., macrophages and vascular endothelium). Host cell receptors also may be present in an age-dependent fashion, accounting for age-related differences in susceptibility

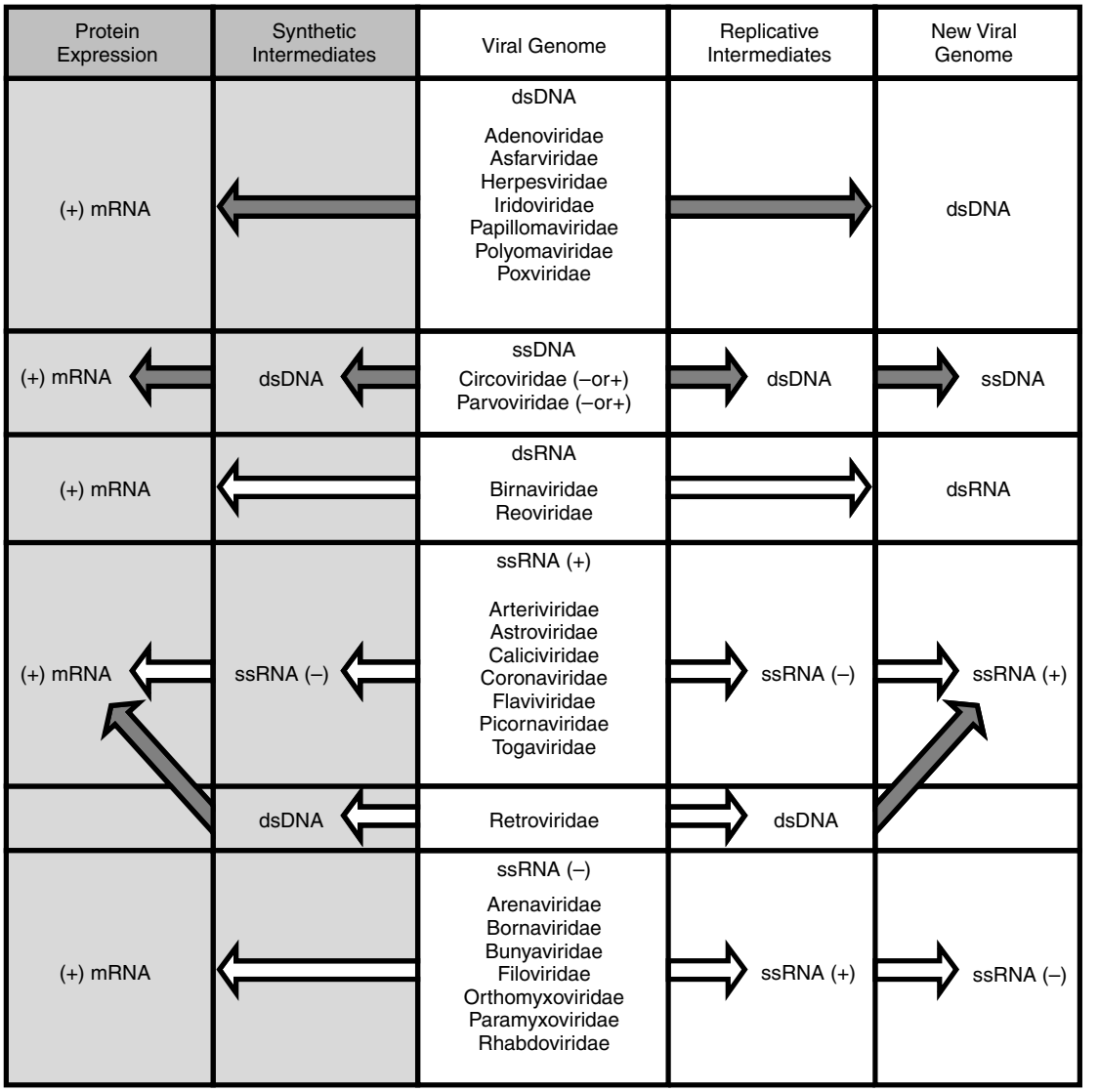

Figure 2.2-3 Summary of the main virus families that infect vertebrates, and general strategies employed by these viruses to produce mRNA for protein expression and to replicate genomes. Required intermediate molecules are indicated. *White arrows* indicate the need for unique viral polymerases, including RNA-dependent RNA polymerase and RNA-dependent DNA polymerase (reverse transcriptase). *Dark arrows* indicate the use of cellular polymerases or viral homologues of cellular polymerases. *ds* = Double-stranded; *ss* = single-stranded; *(+) for RNA* = positive polarity, polarity of RNA used for protein translation; *(+) for DNA* = coding strand, sequence same as for (+) RNA; *(– or +) DNA* = contains single strands of DNA of both polarities.

(Modified from Baltimore D: Expression of animal virus genomes, Bacteriol Rev 35:235-241, 1971.)

to diseases such as polioencephalomyelitis and rotaviral enteritis.[25,26]

Once the viral nucleocapsid gains entry to the cytosol of the cell, the viral genome is released through the process of uncoating. After uncoating, depending on the genetic composition of the virus, the viral genome localizes to the appropriate regions of the cell for replication and mRNA transcription. DNA viruses typically replicate genomes and transcribe mRNA in the nucleus and then transport the mRNA to the cytoplasm for translation. RNA viruses typically replicate, transcribe mRNA, and translate viral proteins in the cytoplasm. These sites of replication account, respectively, for the location of viral inclusion bodies that are diagnostically useful in histopathologic sections. In general, the first viral proteins expressed are regulatory factors, enzymes, and polymerases required for initiation of viral gene expression and diversion of host cell resources to viral replication. Subsequently, viral structural proteins are

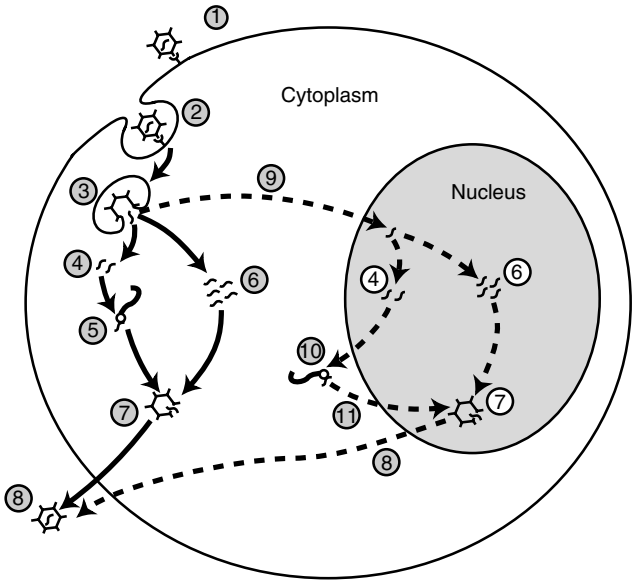

Figure 2.2-4 Schematic representation of the general virus life cycle. Both RNA and DNa viruses share the initial steps, including attachment *(1)*; entry/fusion *(2)*; and uncoating and release of viral genome into the cell *(3)*. *Solid arrows* indicate remaining steps for RNA viruses, which all occur in the cytoplasm, including transcription of mRNA *(4);* translation of virual proteins *(5)*; replication of the viral genome *(6)*; assembly of new virions *(7)*; and release of progeny virions *(8)*. *Dashed arrows* indicate steps for a DNA virus. The life cycle is similar except that the DNA genome is translocated to the nucleus *(9)* for transcription *(4)* and replication of viral genomes *(6)*. Viral mRNA is then translocated back to the cytoplasm for translation *(10)*, and newly synthesized viral proteins are translocated back to the nucleus *(11)* for assembly *(7)*; new virons are then released from both nuclear and cytoplasmic membranes *(8)*.

expressed and assembled with new viral genomes into virions. Enveloped viruses require the additional step of acquiring a lipid envelope by budding the nucleocapsid through areas of the nuclear or plasma membrane into which viral proteins have been incorporated. Viruses then are released, usually in association with death of the host cell, into the extracellular space and blood. Some viruses such as herpesviruses and paramyxoviruses also may transfer progeny virions directly into an adjacent cell by inducing fusion between the two cells, a strategy that allows the virus to avoid contact with neutralizing antibodies in the extracellular space.

VIRUS-CELL INTERACTIONS

After a cell is infected, the process of viral replication may have a number of effects on the host cell. Viruses have evolved a number of strategies and host cell interactions to facilitate their own replication and circumvent host responses that may eliminate the infected cell. In general, virally driven effects favor viral replication, transmission, and persistence and often result in death or dysfunction of the infected cell. Cellular or host responses generally favor control and elimination of the virus from the host. These responses may block viral replication, may initiate innate and specific host immune responses, and are frequently evident clinically as inflammation.

Cell Cycle and Viral Replication

Viral replication requires that the host cell support high levels of nucleic acid replication, mRNA transcription, and protein translation. The growth and division phase of the cell cycle (S phase) is most suited for this purpose. However, many cells infected by viruses already are differentiated fully and in the resting phase (G_0 or G_1 phase). Some of the more complex viruses, such as herpesviruses, poxviruses, and adenoviruses, have virally encoded proteins that activate the cellular systems for growth and division, thus manipulating the cell to create an environment favorable for viral replication. This ability allows tropism for cells that otherwise might not be able to support viral replication. The "immediate early" genes expressed by herpesviruses, which initiate viral replication in differentiated cells, induce progression of the cell cycle from G_0 to the S phase.[27,28] Other viruses, particularly the genetically simpler viruses such as parvoviruses, lack the ability to activate resting cells.[27,29] Thus their tropism is restricted to cells that naturally go through a growth and division phase, such as intestinal crypt cells and hematopoietic cells. Consequently, parvoviral diseases such as feline panleukopenia typically involve replication in the rapidly dividing cells of the intestine (diarrhea) and bone marrow (pancytopenias). If infection occurs in utero during development of tissues such as the cerebellum, neurologic disease also may be observed.

Cell Killing

Cells that replicate virus often are killed as a direct consequence of infection. One mechanism by which viruses kill cells is lysis, often associated with the release of progeny virions. Insertion of viral proteins into cell membranes, budding, direct toxicity of viral proteins, and diversion of normal host cell homeostatic processes to viral replication may result in death of the cell.[15,16,30] Viruses also may activate the cellular self-destruct mechanism of programmed cell death (apoptosis). Although cells may induce apoptosis to try to prevent completion of the virus life cycle, viruses also may use this mechanism to kill the cell and facilitate release of virions.[31]

Neoplasia

Viral infection can cause neoplastic transformation of infected cells. The most common examples in horses include warts (equine papillomavirus) and sarcoids (bovine

papillomavirus). Unlike other species, virally induced invasive neoplastic diseases such as leukemia or lymphosarcoma have not been recognized in the horse. However, these types of diseases may be identified in the future.

Viral proteins that activate the cell cycle into the growth and division phases may lead to neoplastic transformation if expressed in a cell that is not killed by the infection. Papillomavirus infections induce epithelial neoplasms (fibropapillomata) using a virally encoded protein (E5 oncoprotein) that induces proliferation of normally quiescent cells and that presumably is needed for viral replication.[8] The E1A oncoproteins and immediate early proteins of adenoviruses and herpesviruses, respectively, are highly oncogenic in cells that do not allow full virus expression.[32,33] Adenoviral and herpesviral infections in horses currently are not recognized as oncogenic. The lack of oncogenic transformation by these viruses is most likely because abortive infections may not occur in vivo, transformed cells are always removed by the immune system, or the requisite co-factors for transformation are not present. As an example of the role of co-factors, infection of human beings with human herpesvirus 4 (Epstein-Barr virus, the cause of infectious mononucleosis) is not associated commonly with neoplasia in North America or Europe, whereas infection with this virus in Africa is associated strongly with Burkitt's lymphoma and in China with nasopharyngeal carcinoma.[34]

Oncogenic retroviruses, including leukemia and sarcoma viruses, induce neoplastic transformation in several ways but in all cases do so by integration into the host cell genome and activation of cellular oncogenes.[35] Specifically, a retrovirus and its viral promoter sequences may insert upstream from and activate a normally quiescent cellular oncogene in the process of viral gene expression. Other retroviruses, such as the bovine leukemia virus, produce a virally encoded protein, *tax*, that transactivates and upregulates the expression of cellular oncogenes. These are believed to be the primary mechanisms of transformation by the leukemia viruses. Integration of the retroviral genome also may inactivate by insertion host cell repressors required to actively suppress the expression of cellular oncogenes. Some retroviral genomes, such as those of the rapidly transforming sarcoma viruses, may transform the cell by acquiring cellular oncogenes that are then co-expressed along with other viral gene products during replication.

Interference With Differentiated Cell Function

Although not described in horses and still poorly documented in other species such as human beings, some viral infections are theorized to play a role in chronic diseases such as diabetes and obesity. For example, the hepatitis C virus may infect the B cells of the pancreas and interfere with the production of insulin.[11] In experimental infections of rodents, canine distemper virus and Borna disease virus cause obesity, possibly by infection of the hypothalamus and downregulation of leptin receptors.[12] Although the role of viruses in these types of infections is still controversial, advances in the detection of viruses are likely to uncover unusual and previously unsuspected roles for viruses in chronic diseases, including those of horses.

Interactions With Host Immune Responses

One of the main obstacles to successful viral replication in vivo is the host immune response. In response to infection, cells can react in a number of ways to block viral replication, initiate the expansion of specific antiviral immune responses, and target the infected cell for immunologic recognition and destruction. As a result, many viruses have developed counterstrategies to block cell signals that promote these host cell responses.

Interferons induce the expression of a number of cellular proteins that inhibit viral replication in the cell. Secreted interferons similarly impart resistance to viral replication in adjacent cells, and are an important mechanism for controlling the local spread of infection. Some viruses, including paramyxoviruses, adenoviruses, and herpesviruses, produce proteins that interfere with the cell signaling mechanisms required for the expression of interferon-induced proteins.[36,37]

One of the most important systems for recognition of virally infected cells by cytotoxic lymphocytes is endocytic processing of viral proteins followed by expression of the resultant viral peptides on the cell surface complexed with major histocompatibility complex (MHC) class I molecules. Viral products may interfere directly with the processing, transport, or cell surface expression of MHC I molecules or viral peptides, thereby preventing recognition by cytotoxic, $CD8^+$ T lymphocytes.[36,38] In antigen-presenting cells, cell surface expression of viral peptides with MHC II molecules to immune regulatory cells, mainly $CD4^+$ helper T lymphocytes and B cells, is required for initiation and upregulation of antibody and cell-mediated antiviral immune responses. Similar interference with the processing, transport, or cell surface expression of MHC II molecules or viral peptides can interfere with antiviral immune responses.[36] In addition, some herpesviruses also have been shown to interfere with recognition of virally infected cells by natural killer cells, an early, nonspecific cell-mediated immune response.[39]

An interesting and more recently recognized viral mechanism for interference with host immune responses is the use of virally encoded cytokine mimics. Cytokines are critical cell signaling molecules that coordinate and regulate the development of host immune responses. Some viruses, including poxviruses, herpesviruses, and adenoviruses, express cytokine homologs that mimic and interfere with the activity of interferons, interleukin-1,

interleukin-8, interleukin-10, tumor necrosis factor, epidermal growth factor, and granulocyte-macrophage colony-stimulating factor.[36,40] Although the role of these homologs in natural disease is not clear, experimentally they can modulate immune responses and affect disease severity.[36]

Virologic Latency

At the host level, viruses may use several mechanisms to establish persistent infections and avoid immune clearance. Some persistent infections are characterized by continual replication despite the presence of antiviral immune responses. If these types of persistent infections are subclinical, they often are described as clinically latent. However, the viral dynamics at the host level should be differentiated from events at the cellular level. In cells, virologic latency is a specific type of cell interaction and an important mechanism of persistence for some viruses. Virologic latency is defined as the presence of a viral genome that is not producing infectious virus.[41] The genomes of latent viruses also are transcriptionally suppressed and translationally silent so that no viral proteins are expressed that may identify the cell to the immune system as infected. The definition of latency also stipulates that on reactivation, viral gene expression and the production of infectious progeny virions can be resumed, differentiating latently infected cells from cells infected with defective viruses.

The classic latent infection is that of the herpesviruses. For the α herpesviruses, such as EHV1 and EHV4, latent infections are established in the nuclei of sensory neurons and can be maintained indefinitely, and infected animals serve as the reservoir of the virus.[42-44] The only detectable viral gene products in latently infected neurons are a small RNA message called latency-associated transcripts, which are required for maintenance of latency.[41] On reactivation, viral nucleic acids are translocated across synapses to epithelial cells of the nasopharynx, which produce infectious virus. In adult horses the amount of viral replication in the nasopharynx is usually not sufficient to result in clinical disease.[44] The stimuli that induce reactivation are poorly defined, but reactivation can be induced by immunosuppression (e.g., corticosteroids) and presumably by other stressors such as pregnancy, transport, and social stress.[44,45]

Mechanisms of Disease: Virus-Host Interactions

Viral interactions with individual cells are the basis for viral pathogenesis. However, for the whole animal, the severity of disease, or whether infection even results in clinical disease at all, is a much more complex interaction between the classic triad of virus, host, and environment. More specifically, these factors include viral virulence, viral spread within the animal, the intensity of direct and immune-mediated pathologic response elicited by the virus, and the ability of the virus to avoid clearance by the host. Other than the virulence of the virus, which is strictly a property of the virus, the other virus-host interactions can be influenced by the age and genetics of the host and by environmental factors such as stress and nutrition. These factors account for the observation that considerable variation in disease signs can occur among a group of animals infected with the same viral strain.

VIRAL VIRULENCE

Certain strains of a virus are well recognized as causing more severe disease. Although many host factors may influence the severity of clinical disease, virulence per se is strictly a property of the virus. The main properties of a virus that may affect virulence include host cell tropism and replication rate. A tropism change that leads to involvement of additional tissues or facilitates virus spread generally results in more severe disease. The systemic spread of highly pathogenic avian influenza strains described earlier is one example. Outbreaks of EHV1 abortion or neurologic disease strongly suggest that EHV1 strains exist that have a tropism for these tissues compared with EHV1 strains that cause respiratory disease. However, the appropriate studies have not been performed yet to determine the exact basis for this observation. Limited studies of EHV1 genetics and virulence in mouse models have not identified differences between the abortigenic, neurogenic, and respiratory strains.[46] However, the severity of EHV1 respiratory disease in experimentally infected foals can be decreased by deletion of genes that facilitate cell-to-cell spread.[47]

An increase in the viral replication rate usually is associated with an increase in virulence, presumably because of the greater number of infected cells and amount of tissue damage. The virulence of equine infectious anemia virus strains can be correlated directly to plasma virus titers and numbers of infected cells, without any changes in tropism.[48,49] The molecular basis for the increased replication rate is not clear but most likely is caused by variation in viral regulatory sequences and proteins.[50,51]

SPREAD OF INFECTION IN THE HOST

Viral infections generally are regarded as localized or systemic. Localized viral infections are those that are restricted to a single organ system, often at the site of entry. Because infection of the tissue is direct, the incubation period for localized viral infections is usually short, often only a few days. Many infections of the skin or mucosal surfaces are localized, and examples in the horse include infections with enteric rotavirus and influenza. For influenza, virus is inhaled into the

nasopharynx and replicates in epithelial cells of the upper respiratory tract and trachea. Virus is not present in the blood or tissues outside of the respiratory tract. In general, viruses remain localized because they lack the receptors to infect cells of other tissues or circulating cells such as monocytes or lymphocytes that can disseminate the virus. Some viruses are temperature sensitive and remain localized because they are unable to replicate efficiently at core body temperatures. EHV3, the cause of coital exanthema, is restricted to the surface of the genitalia in horses because of its temperature sensitivity.[52] EHV1 and EHV4 are not temperature sensitive, however, and systemic infection may occur with these viruses. The feline respiratory herpesvirus is also temperature sensitive and normally is restricted to the cooler mucosal surfaces. However, hypothermia may lead to dissemination and multiorgan infections.[53] Temperature sensitivity is also a means by which some viruses, such as equine influenza and infectious bovine rhinotracheitis virus, may be attenuated for use as modified-live intranasal vaccines. Infection by the vaccine strain is limited to the cooler mucosal surfaces; the inability to spread systemically prevents sequelae such as abortion and pneumonia.[54,55]

Systemic infections are those in which virus is disseminated to multiple tissues by blood or lymph. This viremia may exist in the form of cell-free virions in the plasma or lymph or may be cell-associated in circulating blood cells, usually monocytes or lymphocytes. The classic paradigm for a systemic infection is infection of mice with ectromelia virus[56] (Figure 2.2-5). Localized viral replication first occurs at the site of entry and in regional lymph nodes. Depending on the level of replication, clinical disease may be present. The virus then enters the blood or lymphatics and spreads to other tissues such as spleen and liver, in which clinical disease may occur. Virus is amplified and then released again for a second, usually higher-titered, viremia that further disseminates the virus to other organs. Each viremic episode is associated with a febrile response and is the basis for the biphasic fever response associated with some viral infections. Because systemic infections require multiple steps, the incubation periods are longer than for localized infections, typically 1 to several weeks. Infections in the horse by eastern, western, or Venezuelan equine encephalitis virus closely follow this paradigm. Localized viral replication occurs at the site of entry (mosquito bite) followed by viremia and dissemination to the central nervous system.[57] For most horses, even nonvaccinated horses, dissemination is controlled before infection of the brain, and neurologic disease is a rare outcome of infection. A variation on the theme is infection of horses with EHV1. The most common clinical disease associated with EHV1 infection is rhinopneumonitis caused by a localized infection of the nasopharyngeal mucosa.[58] In almost all cases a cell-associated viremia also occurs in lymphocytes, but in most infected horses this does not result in disease. However, in some cases, viremia is associated with infection of endothelial cells, and in the pregnant mare vascular damage to the uterus and placenta may lead to abortion.[58,59] Similarly, infection of the vascular endothelium in the central nervous system results in neurologic disease.[60]

Some viruses also may spread in the host through nerves. In the horse, rabies is the best known infection that relies on neural spread. Following local replication in myocytes at the site of entry, usually a bite wound, rabies virus ascends peripheral nerves into the central nervous system, where it replicates in neurons, and then egresses by way of cranial nerves to the salivary gland.[61] EHV1 and EHV4 establish latency in the nuclei of sensory neurons that innervate the nasopharynx and reach the nucleus by ascending nerve axons. Similarly, on reactivation, these viruses egress back down the axon to infect epithelial cells.[43,62]

VIRAL PATHOLOGY

Once a virus reaches a target organ, virally mediated cell death is the fundamental source of pathologic response, disease, and clinical signs observed by the veterinarian. Despite the great complexity of virus-host interactions and the many factors that influence the expression of clinical disease, in actuality viruses have a limited number of ways by which to cause infection. Cells and tissues may be destroyed directly by cytolytic viral infections or by infections that affect the differentiated function of target cells (e.g., neoplasms and immunodeficiencies). Viral infections of organ systems with bacterial flora (e.g., intestinal and respiratory tracts) can disrupt the normal barrier functions of these organs and result in secondary bacterial infections and toxemia that may contribute significantly to the pathologic response. Cell death and pathologic response also may be caused by host immune responses specifically directed against virally infected cells or by indiscriminate inflammatory responses. Virally induced autoimmune diseases have not been described in horses but are another potential source of pathologic response that may be identified in the future.

For most of the clinically important viral diseases of horses, disease manifestation results from some combination of cytolytic infection and immune-mediated tissue destruction. The relative contribution of these mechanisms is primarily a function of viral virulence and host factors that influence the type and intensity of immune responses. The predominant mechanism of pathologic response also can vary with different stages of the same disease, as seen in acute versus chronic equine infectious anemia. In acute disease, most of the diseasea manifestation is caused by direct viral damage and cytokines,

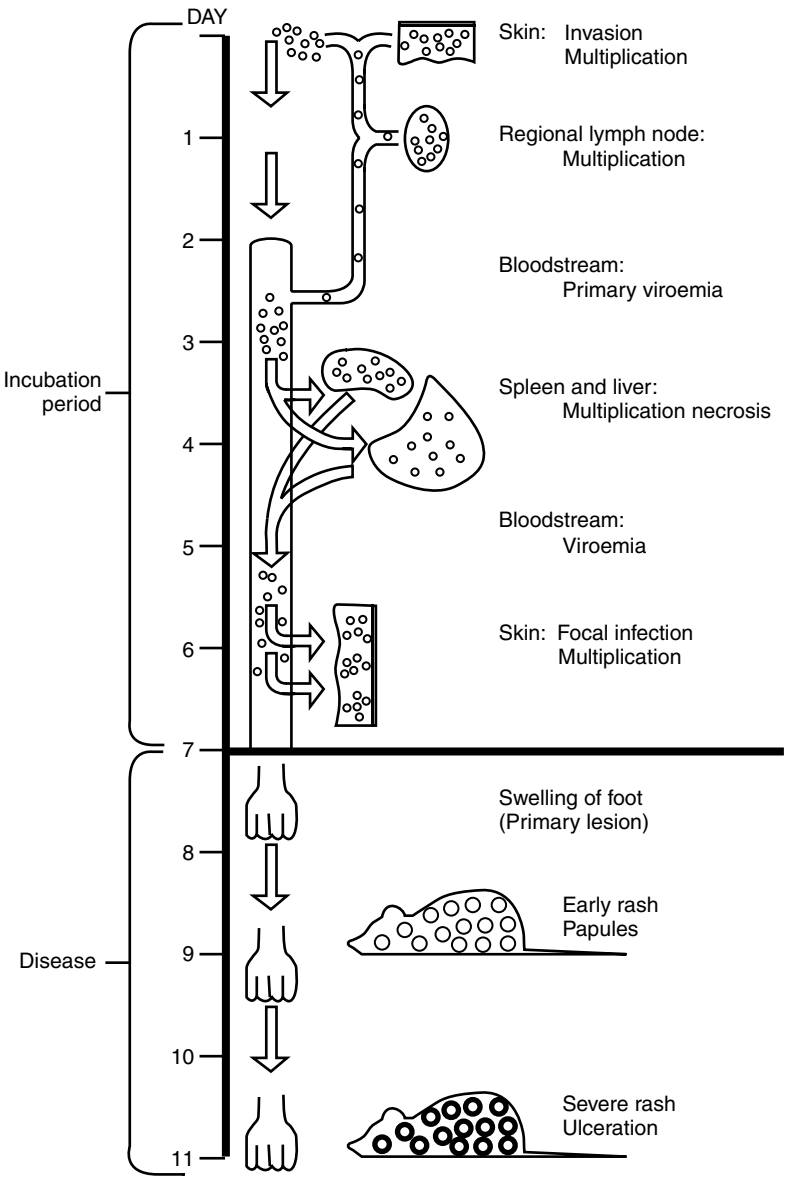

Figure 2.2-5 Schematic representation of the pathogenesis of mousepox (ectromelia), illustrating the classic paradigm for the events in a systemic infection.

(Fenner F: The pathogenesis of the acute exanthems, *Lancet* 255:915, 1948.)

whereas in chronic disease, immune complex–mediated anemia and glomerulonephritis become more significant.

Direct Viral Pathology

The most straightforward cause of damage and disease in an organ system is for the virus directly to kill or cause dysfunction in the infected cells. As described previously, cell death may be caused by cytolysis resulting from viral replication and interference with normal cell homeostasis or by initiation of apoptosis. Cell dysfunction usually is observed as neoplastic transformation, although more vague effects on other differentiated cell functions, such as insulin secretion, remain possibilities.

The disease associated with a given viral infection is related to the affected organ system(s), the number of cells destroyed, and the sensitivity of the affected organ system to dysfunction. If the number of infected cells is not sufficient to lead to clinically significant organ dysfunction, then the result is a subclinical infection. Vaccination and naturally acquired immunity are important factors that limit the number of infected cells. However, epidemiologically these infections may still be

important because these animals may still be infectious. When enough cells are infected to lead to overt organ dysfunction, then clinical disease becomes apparent. The threshold for clinically significant damage varies considerably between organs and the types of cells. EHV1 infections of the respiratory epithelium, which has a large number of cells with a high turnover rate, produce mild clinical disease even with a high rate of infection. On the other hand, much more significant clinical manifestations of EHV1 infection such as abortion and neurologic disease are caused by infection of a few endothelial cells because minimal vascular damage can lead to thrombosis, ischemic necrosis, and damage to large amounts of tissue. If viral infection results in neoplastic transformation of a cell type, then disease may progress according to the characteristics of the neoplasm, whether or not the virus remains associated with the tumor.

Of particular importance are infections of the respiratory and intestinal tracts that contain normal bacterial flora and have important barrier functions to prevent access of bacteria and toxins to deeper tissues. Consequently, complications of bacterial infection and toxemia are important secondary problems of viral infections.[63,64] For example, upper respiratory viral infections damage the ciliated epithelial cells that function to move mucus and other respiratory secretions outward and provide a barrier between the mucosal bacterial flora and the underlying subepithelial tissues. Damage to this barrier may lead to opportunistic bacterial pneumonias by normal bacterial flora such as *Streptococcus equi zooepidemicus* or *Mannheimia haemolytica* in horses or cattle, respectively.

Immune-Mediated Viral Pathology

In most viral infections, immune-mediated pathologic response contributes significantly to disease and in some cases may be the predominant cause of disease manifestation. This paradigm has been documented elegantly in classic experiments with lymphocytic choriomeningitis virus infections of mice in which immunodeficient mice develop persistent infections but do not develop disease and do develop disease if their immune systems are reconstituted.[65] Details of equine immune and inflammatory responses are covered in detail in Chapter 1 and are described in this section only in general terms and as they relate to viral infections.

The primary antiviral immune responses initiated in viral infections include natural killer cells, virus-specific cytotoxic ($CD8^+$) T lymphocytes, and to a lesser extent antibody-mediated lysis of infected cells (antibody-directed cellular cytotoxicity, complement fixation, and phagocytosis by macrophages and neutrophils). These immune responses detect and selectively kill virally infected cells and are a significant cause of cell death. Indiscriminate inflammatory responses also may kill uninfected cells in the vicinity of infected cells, primarily from activation of monocytes and granulocytes with release of a wide array of cytotoxic molecules (e.g., lysozyme, proteases, lipases, and oxygen radicals). Secondary bacterial infections greatly exacerbate indiscriminate tissue damage, not only from the direct effect of bacterial toxins but also because of extensive recruitment of neutrophils and complement, which are highly nonselective components of the antibacterial immune response.

Antibody-mediated immune complex hypersensitivity reactions also may play a major role in the genesis of viral disease. Antibodies bound to soluble viral antigens fix complement and opsonize neutrophils, again leading to indiscriminate cell and tissue destruction. Viral antigens may be on the surface of cells, such as erythrocytes or platelets, resulting in immune-mediated anemia and thrombocytopenia, respectively.[66] When viral antigen is in excess relative to antibody, circulating immune complexes are formed that can be deposited in the capillary beds of tissues such as joints and renal glomeruli. In chronic equine infectious anemia virus, with persistent viral replication and antigenemia in the presence of antiviral antibodies, these mechanisms are primarily responsible for the characteristic lesions of anemia, thrombocytopenia, and glomerulonephritis.[66,67] Nonneutralizing antiviral antibodies actually may serve to increase the number of infected cells and the severity of disease through the mechanism of antibody-dependent enhancement. This enhancement occurs when antibodies bind to the virus and facilitate attachment and entry into Fc receptor–bearing cells such as macrophages by serving as a form of alternative receptor.[68] Antibody-dependent enhancement has been shown to be involved in the pathogenesis of a number of diseases, including feline infectious peritonitis,[69] human respiratory syncytial virus,[70] and dengue hemorrhagic fever.[71]

Soluble inflammatory mediators released by infected cells and inflammatory cells in response to viral infections are also significant contributors to clinical signs and disease manifestation. These mediators include a wide array of cytokines, interleukins, and other proinflammatory molecules with potent systemic and local effects. The most evident of these is the febrile response caused by interleukin-1 released by macrophages in response to virally infected cells. Increased levels of other soluble factors, including interferons, interleukins, transforming growth factor, and tumor necrosis factor are also present in many viral infections.[72,73] These cytokines are primarily proinflammatory and immunoregulatory and are generally important for the control of viral infections but also can be responsible for many of the nonspecific clinical signs of viral infection such as depression, malaise, and in chronic infections, cachexia. Soluble mediators also may have important local effects. Vasoactive factors

such as prostaglandins and leukotrienes cause edema and swelling. In equine infectious anemia virus, tumor necrosis factor α, transforming growth factor β, and interferon-α have been shown to suppress hematopoiesis and contribute to the development of anemia and thrombocytopenia.[74]

Autoimmunity

Although not described in horses, viral infections in other species can induce immune-mediated responses to host cell antigens and autoimmune diseases. The best documented human autoimmune disease suspected to be initiated by viral infections is the Guillain-Barré syndrome, in which infection with cytomegalovirus or Epstein-Barr herpesvirus elicits antinerve ganglioside immune responses and demyelinating disease.[75] Postinfluenza myocarditis is an occasional sequela in horses and human beings and is a potential autoimmune disease. Although no direct evidence supports this theory, influenza virus is not identified consistently in affected heart muscle and the pathogenesis is not known.[76]

IMMUNE AVOIDANCE

A key requirement for viruses to be maintained in nature is to persist successfully in a reservoir host (if the reservoir is an infected animal) and to be transmitted to another susceptible host. One of the most important obstacles to persistence and transmission is detection and elimination by the host immune system. Transmission to a new host also may require that the virus avoid preexisting immunity from prior natural exposures or vaccination. Within the host, rapidly replicating viruses such as influenza may shed and transmit virus before the host can mount specific antiviral immune responses. Herpesviruses avoid detection during latency by not expressing any viral proteins. For persistent viral infections that continually replicate within a host, such as retroviruses, evasion of developing immune responses is necessary. Immunodeficiency viruses may cripple antiviral immune responses by directly infecting immunoregulatory $CD4^+$ T lymphocytes. As described previously, many other viruses can dysregulate host immune responses by expressing cytokine mimics.

One of the most important mechanisms of immunologic avoidance is antigenic variation in which neutralizing viral antigens are altered so that they are no longer recognized or accessible by host immune responses. The most important of these antigens includes viral proteins bound by neutralizing antibodies (e.g., virus receptors) and any peptides presented in the context of MHC I or II cell surface molecules for recognition by cytotoxic ($CD8^+$) T lymphocytes or helper ($CD4^+$) T lymphocytes, respectively. Antigenic variation is generated by nucleotide errors during transcription or replication, which result in amino acid substitutions in the relevant epitopes. This process is facilitated by viral polymerases that are inherently error-prone and lack proofreading functions and by placing the sequences for the relevant epitopes adjacent to other genomic sequences or structures that further predispose to transcriptional errors.[19] Other mechanisms by which some viruses may modify their antigenicity is through intramolecular recombination/duplication or reassortment of segmented genomes (e.g., influenza and African horse sickness).[19,21] For reassortment, co-infection of a single cell with genetically different virions may result in a progeny virion with segments derived from both virions and a major change in antigenicity. In influenza these are called antigenic shifts, and the radical change in the antigenicity of the virus may render preexisting immunity in the host population ineffective at preventing outbreaks of disease with high morbidity and mortality.[21] Viruses also may facilitate the production of nonneutralizing antibodies that sterically interfere with the ability of neutralizing antibodies to bind. Although the mechanism of antigenic variation is primarily random, immunologic selection determines which variants successfully emerge and are capable of replicating within the host or the host population.

HOST GENETICS

Genetic differences in susceptibility to disease have been well documented. In an outbred population of animals, the considerable variation in the type or severity of clinical disease is well recognized, even when animals are infected with the same virus strain and have no recognizable differences in other factors such as age, challenge dose, nutrition, and general health status. Conversely, highly inbred populations may be more uniformly susceptible to a viral disease.[77] Thus inbreeding can pose problems for endangered species, such as Przewalski's horse, or other populations with limited genetic variability, which may incur high rates of morbidity or mortality if the animals are infected with a virulent virus.

Although in many virus-host interactions the basis for genetic resistance to disease is not well defined, host genetics have been shown to affect the tropism of the virus and influence the type and intensity of immune responses to a viral infection. In human immunodeficiency virus infections, genetically determined absence or presence of certain co-receptors has a significant effect on susceptibility to disease.[78,79] Immunologically, host genetics defines the repertoire of antigen-specific recognition molecules (antibodies and T cell receptors) and antigen-presenting molecules (MHC I and II). Consequently, genetically different animals respond to different subsets of viral antigens. The relative inability to react to a neutralizing epitope prevents or delays effective control of viral replication.[80] Different subsets

of viral antigens may favor the development of a type 1 T helper cell (cell-mediated) or type 2 T helper cell (antibody-mediated) immune response, which has implications for the ability to control infection and for immune-mediated pathologic response.[81-83]

Advances in the Diagnosis of Viral Diseases

The diagnosis of viral infections traditionally has relied on the detection of antiviral antibodies by serologic tests and the direct demonstration of virus by isolation in cultures of living cells or in laboratory animals. Although these principles are still the foundation for the diagnosis of viral diseases, recent molecular biology advances have improved the clinical use of viral diagnostics greatly.

The sensitivity and specificity of antibody testing has been enhanced by the recombinant viral proteins or peptides for use as antigens in serologic tests. One example is the use of recombinant antigens in antibody tests for equine infectious anemia virus. The replacement of whole-virus antigen preparations with recombinant viral proteins has improved the sensitivity and specificity of the agar gel immunodiffusion (Coggins) and enzyme-linked immunosorbent assay tests.[84,85] The improvement in specificity is especially important when screening for a low-prevalence disease in which the predictive value of a positive result is low, as is the case for equine infectious anemia in the United States.

However, the greatest advances have been in the area of detection of virus in clinical specimens. The clinical utility of traditional methods of virus isolation in cell culture often is limited by the sensitivity, specificity, predictive value, or the length of time needed to perform this type of assay. Another significant limitation of virus isolation, particularly for the more labile enveloped viruses, is the requirement for viable virus, which may be compromised by autolysis or transport to the laboratory. In many diagnostic laboratories, virus isolation in cell culture has been replaced by immunohistochemistry and the polymerase chain reaction (PCR), both of which are sensitive and specific tests that can be completed within a day or two. Immunohistochemistry is a method that detects viral antigens in formalin-fixed tissue samples. Although immunohistochemistry technology has been available for many years, this assay has become more widely used because of improvements in the methods for antigen retrieval and the greater availability of antiviral monoclonal or polyclonal antibodies. The aldehydes in formalin cross-link proteins during the fixation process and prevent the recognition of epitopes by antibodies. For many viruses, reliable protocols now have been established that use carefully controlled protease digestion of the tissue sections so that the availability of epitopes is restored.[86] The tissue sections then can be incubated with antiviral antibodies, and bound antibodies can be visualized with a variety of colorimetric systems (Figure 2.2-6). The specificity of immunohistochemistry depends highly on the antiviral antiserum and has been improved greatly by the availability of antiviral monoclonal antibodies and recombinant viral proteins that can be used to generate monospecific antisera.[87-89]

The polymerase chain reaction (PCR) assay is a nucleic acid amplification method that detects viral DNA or

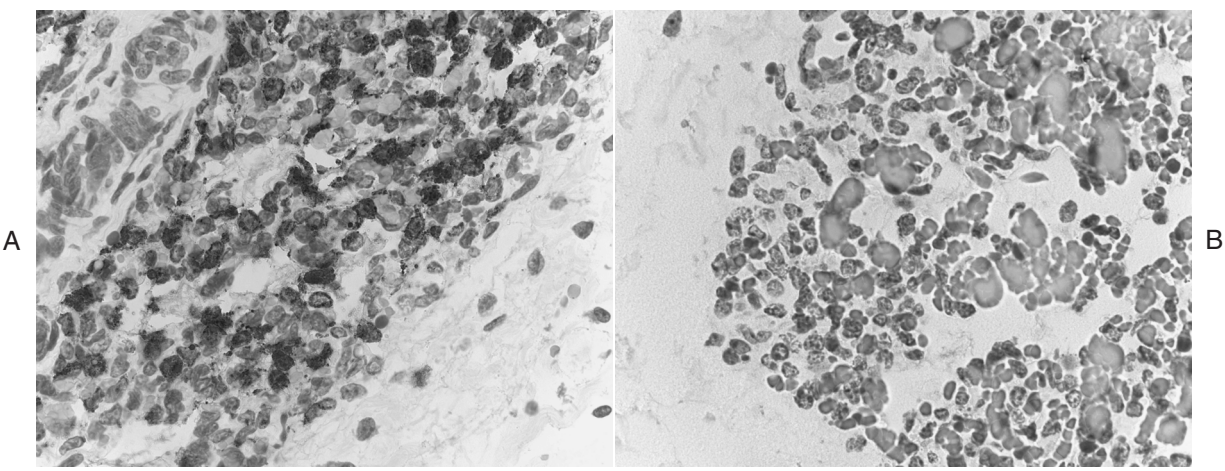

Figure 2.2-6 Immunohistochemistry for equine herpesvirus in tissues of an aborted equine fetus. Panel A is a section of lung stained with anti-EHV antibodies. Bound antibodies are visualized with a secondary antibody conjugated to horseradish peroxidase that when reacted with the chromogen develops a visible precipitate in the cytoplasm of infected cells. Panel B is a negative control, which is a section of lung from the same fetus stained with an isotype-matched antiserum that does not contain anti-EHV antidodies; no precipitate is visible.

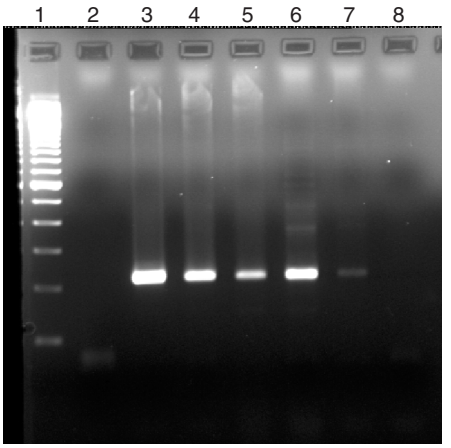

Figure 2.2-7 Analysis of PCR reactions for equine infectious anemia virus. PCR products are electrophoresed in an agarose gel. DNA in the gel is stained with ethidium bromide and visualized with ultraviolet light that causes the DNA to fluoresce and appear as white bands in the gel. Lane *1* is a size standard to determine the size of the DNA amplicons. Lane *2* is a negative control, and the lane *3* is a positive control. The samples in lanes *4, 5, 6,* and *7* are positive; the sample in lane *8* is negative.

RNA extracted from fresh or formalin-fixed tissue samples. Only a few years ago the techniques used to purify nucleic acids and perform the PCR reaction were limited by expense and practicality to the research laboratory. However, the simplification and commercialization of these procedures has made this type of test economical and clinically useful for diagnostic laboratories.[90,91] The PCR test uses oligonucleotide primers and a thermostable DNA polymerase (Taq polymerase) to amplify exponentially the viral nucleic acids. The basis for specific amplification in the PCR assay is the sequence of the oligonucleotide primers, which are produced synthetically to be complementary and thus bind to the viral nucleic acid sequences. To produce complementary primers, the sequence of the viral DNA or RNA must be known. Consequently, this assay is only useful for testing for viruses that have been sequenced, and in the past, sequence data limited the ability to design PCR primers. However, because of advances in the technology for sequencing and database management, virtually all of the significant equine pathogens now have complete sequence data available. At the end of the PCR procedure, the amplified nucleic acids, that is, a positive result, are visualized most commonly with agarose gel electrophoresis in which the amplicons in the gel are viewed with a fluorescent dye, and the product presumptively is confirmed as the correct one based on its molecular size (Figure 2.2-7).

Immunohistochemistry and PCR are now routine tests that generally are sensitive, specific, and rapid. However, care must be taken not to make assumptions about the performance characteristics of new tests. For example, although PCR assays are often more sensitive than virus isolation, this is not automatically true. The sensitivity and specificity of any new test must be evaluated individually and with respect to the performance of the previous test. Also, the significance of any virus identified in a specimen still must be interpreted in the context of the pathogenicity of the virus and clinical disease, regardless of the method used to detect the virus.

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2.3—Internal Parasite Infections

Maureen T. Long

Horses serve as hosts for numerous parasites that induce a wide range of pathologic and immunologic responses,[1] including hypersensitivities and other forms of immunopathology. Immune responses leading to protective resistance against reinfection occur, but the level of this resistance is most often incomplete. Mechanisms associated with these responses have not been investigated extensively in the horse, but information is available from other host-parasite systems that is relevant to the horse. The purpose of this section is to acquaint the reader with contemporary thoughts on host-parasite interactions. Because of their prevalence, major importance to equidae, and information available, coverage is limited to helminth parasites that occur in most developed and nontropical countries.

Parasite-Induced Lesions

Infection with most metazoan parasites results in inflammation and structural and functional changes of the organs invaded, the outcome being an alteration of the host's physiologic state. The degree of alteration depends on the existing physiologic condition of the animal, which is dictated to a great degree by its age, nutritional status, and previous immunologic experience with the parasite. The numbers of parasites introduced and the specific parasite also affect the degree of physiologic change that occurs. When these factors favor major alterations, the results are readily identifiable clinical signs of infection. Subclinical infections, although less apparent, are potentially important to the general health of the animal and continued transmission of the agent. The pathophysiologic effects of infection by ectoparasites, helminths, and microorganisms are in many cases similar. Abnormalities in weight gain, skeletal growth, reproduction, and lactation may result from infections with any of these agents. These changes often are related directly to parasite-induced anorexia, disruption of metabolic processes, and anemia. An understanding of the morphologic and biochemical lesions produced by specific parasites clarifies the role of these agents in clinical and subclinical conditions associated with the infections. A majority of detailed studies on the pathophysiology of parasitic infections have been conducted in laboratory animal models and domestic

The authors acknowledge and appreciate the original contribution of Thomas R. Klei, whose work has been incorporated into this section.

animal species other than the horse.[2] However, the classical pathologic effect of parasitic infections of the horse has been reviewed.[3,4] The following discussion outlines some recent observations on host-parasite interactions that may be significant to equine medicine. Examples of host-parasite interactions responsible for alterations in host homeostasis are presented as they relate to the gastrointestinal tract, lungs, and skin.

GASTROINTESTINAL TRACT

Internal parasites are most important to equine health as mediators of gastrointestinal symptoms such as colic and diarrhea. Although almost all internal parasites have been implicated inferentially as causative agents of colic at some time, large strongyles, principally *Strongylus vulgaris* and to a lesser extent *Parascaris equorum,* classically have been considered major pathogens. Details of the pathogenesis of colic associated with migration of *S. vulgaris* through the mesenteric arteries and the resultant thrombosis, infarctions, and necrosis of the intestine have been described in detail elsewhere.[3,4] Although this condition has been well described, some points are particularly noteworthy. Histologic studies of experimentally infected parasite-free pony foals during the initial stages of the infection indicate that the severity of the lesions produced in the intestine cannot be attributed solely to mechanical disruption caused by larval migrations and that these larval stages induce some biologic amplification system within the mucosa, which results in the degree of inflammation observed.[5] Although the mechanisms involved in this response have not been investigated, the histologic nature of the lesion is characteristic of an Arthus reaction, suggesting an involvement of the immune response. Other experimental studies using the parasite-free pony–*S. vulgaris* system have implicated the immune response in the mediation and regulation of the arterial lesions produced by this parasite. Passive transfer of immune serum, but not normal serum, reduced the severity of arteritis and clinical signs associated with experimental infections without reducing the numbers of parasites that developed in these ponies. However, treatment with immune serum also induced an anamnestic eosinophilia and significant perivascular infiltration of eosinophils in the cecum. The reduction in intravascular lesions may have been associated with an inactivation of parasite-secreted inflammatory factors by antibody or serum enzymes. This serum also may have contained nonspecific host-derived antiinflammatory substances. The exacerbation of the eosinophil response may have been associated with the formation of immune complexes. Although the mechanisms are unknown, the results suggest that the immune response may simultaneously modulate and potentiate inflammation. Larvicidal treatment of *S. vulgaris*–infected horses and killing of intravascular larvae has been postulated to release a bolus of antigenic factors from these larvae within the mesenteric vasculature, resulting in an exacerbation of arterial and intestinal lesions and colic. Experimental testing of this hypothesis indicates that this phenomenon does not occur and further that viable larvae are necessary to maintain the arteritis and eosinophilia seen.[6,7]

P. equorum–associated colic in foals has been related to intestinal impaction and rupture and is not considered to be of major significance in adult horses.[8] However, ascarid nematodes are particularly potent sources of allergens, and conceivably the hypersensitized mature horse may respond to low-level infections by this parasite. Observations made in the author's laboratory are noteworthy in this regard. Two mature *Parascaris*-free adult horses were inoculated intradermally with less than 90 µg of saline-soluble somatic extract of adult *P. equorum* to test for immediate hypersensitivity to this antigen. Both horses experienced an immediate systemic response and colic. One of the horses died within 3 hours of intradermal inoculation. Necropsy results were consistent with the diagnosis of colitis X. Because of the allergic potential of ascarid nematodes and the sensitivity of the equine gut to immediate hypersensitivity reactions, this potential is worthy of further characterization and consideration.

Recent clinical observations have piqued an interest and concern over the pathogenic potential of infections by *Anoplocephala perfoliata*. Case reports have described cecal ruptures and intussusceptions of the cecum and colon associated with these infections.[9,10] However, detailed retrospective analysis of the concomitant occurrence of intussusception and colic and *A. perfoliata* infections has failed to demonstrate any causal relationship between these conditions and tapeworm infections.[11] These parasites inhabit the region of the ileocecal junction and produce ulcerated lesions of the mucosa and submucosal inflammation. However, the parasites are common, and possibly the association of tapeworm infections and clinical signs attributed to them are caused by chance alone. Detailed experimental investigations of these infections have not been conducted, and thus specific details on the pathogenesis and relevance of these lesions are lacking. However, an association between tapeworm infections and colic of ileocecal origin has been suggested in a case-control study of 231 horses.[12]

Until recently, cyathostomes (small strongyles) have not been considered of major importance, particularly as causative agents of colic. In this regard the field studies of Uhlinger are of particular importance.[13] In these controlled experiments, different anthelmintic treatment regimens were used to test their efficacy in reducing the incidence of colic. The more efficacious treatment programs significantly reduced the incidence of colic by 2 to 13 times that seen in the same herds before

implementation of the more efficacious treatment. Because of the management programs used before the initiation of this study and the results of fecal cultures, one can assume that the primary parasites present in these horses were cyathostomes. These data strongly implicate a role for cyathostomes in a substantial proportion of colics observed under field conditions. The parasite or host factors involved in these colic cases are unknown.

Cyathostomes have been implicated in numerous case reports with seasonal diarrhea in adult horses, which is a condition called larval cyathostomiasis. These cases are characterized by a sudden onset of diarrhea during the late winter or spring. Mature horses usually are affected, and infections are often fatal. The condition is difficult to diagnose, and the only consistent signs are weight loss and diarrhea.[14] Large numbers of larval cyathostomes are found in the feces or in intestinal contents and within the mucosa of these horses. These symptoms are related to the synchronous emergence of fourth-stage larvae of these parasites from the mucosa. These larvae build to potentially large numbers within the mucosa because of the arrested development of infective larvae. The seasonality of the occurrence of this condition at present does not appear to vary in different climatic regions as does the analogous bovine condition of type II ostertagiasis. Specific parasite or host factors associated with the regulation of the hypobiotic state of the larvae or the inflammatory response initiated at parasite emergence have not been described.

In view of the paucity of specific mechanistic information on the pathophysiologic effects of equine gastrointestinal parasites, a synopsis of relevant information gathered from other model systems is warranted.[2,15] Parasitic organisms may induce changes in gastrointestinal function directly by mechanical disruption of tissues and cells or by the release of factors that directly alter cell function. Induction of the immune response serves as an anamnestic amplification system. The result of these changes is an alteration in function of the smooth muscle and epithelium of the bowel. A number of helminth parasites, including *P. equorum,* have been demonstrated to produce intestinal smooth muscle hyperplasia. Evidence suggests that this response may be induced by intestinal inflammation or stenosis associated with parasitism. Contractility of these muscles also has been demonstrated to be induced in a Schultz-Dale reaction by stimulation with parasite antigens. This response is mediated in rats by mast-cell–derived 5-hydroxytryptamine and in guinea pigs by histamine. A regulatory relationship of myenteric neurons to these antigen-induced changes also has been demonstrated in this model system. These latter experiments suggest that antigen-induced stimulation of smooth muscle contractility may be blocked correspondingly by γ-aminobutyric acid similarly stimulated by mast cell products. This complex system may be an adaptation by the host to maintain homeostasis in the face of continued antigenic stimulus. What is noteworthy is that strongyle-induced alterations in myoelectric activity of the equine small intestine and colon have been demonstrated in vivo.[16,17] In some of these experiments, dead *S. vulgaris* larvae evoked an alteration of the smooth muscle response in previously exposed ponies, suggesting a role for the immune response in the stimulation of the hyperactivity.[18]

A recent series of in vitro and in vivo experiments on the direct interaction of factors produced by the adult heartworm *Dirofilaria immitis* and arterial endothelium are exciting and noteworthy.[19] The results of these studies indicate that endothelial cell–mediated vasodilation is depressed in the presence of small (100 to 1000 molecular weight) parasite-derived pharmacologically active substances. Seasonal pathologic changes in infected arteries in dogs have been associated with the release of these substances by adult parasites. The nature of the factor or factors involved is unknown at this time. Production of similar factors in the mucosa or mucosal vasculature by nematodes could induce focal alteration in blood flow and other physiologic effects on smooth muscle. In general, these studies demonstrate the potential variety and complexity of metazoan-host interactions that have yet to be defined.

Many parasites and other infectious agents alter the structure of the intestinal epithelium, causing villous atrophy and crypt hyperplasia. Hypersensitivity responses are linked to functional changes in epithelium, such as decreases in epithelial brush border digested enzyme activity, decreased absorption, reduction in fluid absorption, and an increase in fluid secretion.[2] In vitro studies on the Cl^- secretion of isolated intestines from guinea pigs and rats infected with the nematode *Trichinella spiralis* have proved to correlate with in vivo fluid secretion.[15] In this system, antigenic stimulation induces an increase in Cl^- secretion similar to that induced by cholera toxin. The mechanism of this phenomenon in rats is mediated by antiparasite immunoglobulin E–mast cell release of at least two pharmacologically active substances that function in two phases. The immediate response depends on T cell–mediated mast cell hyperplasia, which is important in the release of 5-hydroxytryptamine and histamine. These factors act on enteric neurons to induce the immediate increases in Cl^- secretion from epithelial cells. The second phase is independent of mast cell hyperplasia and is mediated through 5-hydroxytryptamine and histamine–initiated increase in de novo mucosal synthesis of prostaglandin, which mediates a second phase of secretion. These in vitro phenomena can be blocked at various stages by extrinsically supplied pharmacologic agents. The suggestion is that similar levels of control may be

active in vivo, mediating these effects and maintaining homeostasis in most cases. Although studies of this nature have not been conducted with equine tissues or parasites, similar mechanisms and activities likely occur.

RESPIRATORY SYSTEM

Several nematode parasites infect the equine lung. These include migrating stages of *P. equorum* and *Strongyloides* en route to the small intestine. Migrating stages of aberrant parasites, such as *Habronema* sp., *Draschia megastoma,* and *Strongylus* spp., which induce granulomatous foci in the lung parenchyma, and adults and larvae of the lungworm *Dictyocaulus arnfieldi,* which inhabit the bronchi, also occur. Host responses to two of these are noted.

P. equorum larval migrations in the lungs of yearling horses produce more severe clinical signs and inflammatory responses than in foals reared parasite-free. These infections in yearlings are accompanied by focal accumulations of lymphoid tissue, indicating an induction of an active local immune response. The reaction suggests that this is an age-related phenomenon.[8] However, more severe reactions likely could result from previous sensitization to *P. equorum* antigens. Increased responses of this nature have been described in the livers of pigs immunized with *Ascaris suum* antigens following challenge infections.

Dictyocaulus arnfieldi infections of donkeys rarely produce clinical signs, and these equidae have been suggested to be the natural host for this parasite. Infections of horses produce more severe and prolonged bronchial inflammatory responses similar to *Dictyocaulus* sp. infection in other hosts. The mechanisms associated with this differential response have not been defined but are common in unadapted host-parasite associations. Possibly the more significant inflammatory reaction of the horse to these parasites is caused by the absence of down-regulatory mechanisms that are established in the more adapted natural host, the donkey.

SKIN

Reactions to the filarial nematode *Onchocerca cervicalis* illustrate variations seen in responses to chronic parasite infection. Focal, alopecic, depigmented, pruritic lesions often are seen in infected horses. Not all infected horses react to this infection, and the appearance of clinical signs is often seasonal. Detailed studies have not been conducted on the pathogenesis of these lesions in horses. However, similar conditions occur in human onchocerciasis,[20] and the host-parasite responses active in human beings likely are also present in the horse. Lesion development is associated with immune-mediated killing of microfilariae in the skin. Parasites appear to be killed in an antibody-dependent cell-mediated reaction. In this response, antimicrofilarial surface immunoglobulin G and E antibodies mediate adherence and degranulation of granulocytes, which are predominantly eosinophils. The major basic protein of eosinophils has been demonstrated in the tissues of patients with dermal lesions, and eosinophil toxic enzymes and proteins are responsible for many of the changes seen. The reason for the absence of these lesions in most horses is unclear. Human onchocerciasis and filariasis are spectral diseases in which regulation of immune responses has been associated with the lack of pathologic responses to the parasites.[21] Immune regulatory mechanisms associated with these infections include immune tolerance, anergy, induction of immune regulatory circuits involving suppressor T cells or macrophages, and most recently the potential shift in T helper cell subsets, which potentially alters the production of specific cytokines during different phases of the infections. The parasites themselves also may play a role in these regulatory responses. High-molecular-weight proteins and phosphorylcholine-containing proteins isolated from filariae have been demonstrated to suppress lymphoproliferation in vitro.[22] Although the activity of these proteins is yet to be demonstrated in vivo, chemotherapeutic elimination of circulating microfilaria from infected individuals restores previously suppressed parasite-specific lymphocyte responses. The presence of these types of parasite-associated immune regulatory events has yet to be studied critically in the horse. However, the seasonal variability in skin responses to the *Onchocerca* microfilariae by horses in some regions has been investigated. In this instance, the onset of ventral-midline dermatitis during the summer may be related to a seasonal fluctuation in microfilarial burdens of the skin, which also peak at this time.[23] Not only do total numbers increase, but also the microfilariae are found more commonly in the surface layers of the skin. Interestingly, this period of abundant microfilariae corresponds with the seasonal peak in numbers of the vector *Culicoides varripennis.* Although speculative, correlations in the peak availability of microfilariae and vectors may be an evolutionary adaptation by these parasites to maximize transmission and survival of this parasite species.

Protective Resistance

Resistance to infection may be innate or acquired. In some instances, innate resistance to equine parasites has been attributed to age, with older individuals being resistant. Most equine helminth parasites only develop in the horse, and conversely the horse exhibits an innate resistance to most nonequine parasites. Exceptions to this rule are parasites with a broader host range that occasionally infect horses, such as larvae of the tapeworm *Echinococcus granulosus* and the liver fluke *Fasciola hepatica.*

Trichostrongylus axei, a parasite of ruminants, establishes readily in the equine stomach and only produces significant lesions when present in large numbers. In some cases, parasites that develop in the horse induce more severe lesions and clinical signs than in their apparent normal host, as has been described for *D. arnfieldi.*

Age resistance to *P. equorum* and *S. vulgaris* has been described to occur in horses by comparing susceptibility of young and old ponies reared under parasite-free conditions. Apparently the reaction of the lung to migrating *P. equorum* larvae is more significant in mature horses and suggests that an immune response occurs in this site.[9,10] Initial reports on age-acquired resistance to *S. vulgaris* infection have not been substantiated by further experimentation, and these results remain equivocal.[24]

The occurrence of acquired resistance to equine parasites can be inferred from the observation that older, chronically exposed horses generally have lower burdens of parasites than do similarly exposed young horses. With these criteria, acquired resistance is apparent to infections with *Strongyloides, P. equorum, Strongylus* spp., and cyathostome species. Extensive experiments are limited, however, to those on *S. vulgaris.*

The level of resistance acquired in most cases is partial and of a concomitant type; that is, some stages of the parasite, such as arterial larvae of *S. vulgaris,* may reside within the horse in the face of an active acquired resistance against newly acquired infective stages. Resistance to infection with *S. westeri* adult parasites is inferred by the short duration of their life cycle within the small intestine and the failure of subsequent exposures to establish patent infections. Mares, however, remain infected with arrested third-stage larvae, which subsequent to foaling are transmitted to the foals in milk four days postpartum. Although not studied in horses, similar phenomena occur in swine strongyloidosis. In these infections, an apparent protective resistance against the migrating stage L_3 parasite occurs that is effective in preventing reestablishment of the intestinal infection but is ineffective against L_3 parasites, which are sequestered in the abdominal fat of the sow.[25] Similar epidemiologic phenomena occur in *S. westeri* infections of horses implying that similar immunologic mechanisms are also active.

Immunologic mechanisms associated with protective resistance are presented primarily as they relate to parasites that inhabit the lumen of the gastrointestinal tract and secondly as those that undergo extensive extraintestinal tissue migration.

GASTROINTESTINAL PARASITES

Immune responses directed toward gastrointestinal nematodes vary significantly among hosts and against different parasite species within a given host.[22] However, some generalities may be stated that may serve as a background for understanding these responses in the horse. A phenomenon termed *self-cure* has been described in sheep, in which the ingestion of significant numbers of infective larvae induces the expulsion of existing adult parasites. This expulsion is initiated by a species-specific immediate-type hypersensitivity response that may cause the nonspecific expulsion of other nematode species. Although this phenomenon has not been examined in the equine, experimental infections of naturally parasitized ponies with large numbers of *S. vulgaris* L_3 induced a dramatic decrease in preexisting strongyle fecal egg counts, suggesting that a self-cure–like reaction may occur under some conditions.

More typically, establishment of primary infections results at some time in spontaneous expulsions of these worms because of senility or, as demonstrated in laboratory animal model systems, active acquired immune responses. This phenomenon occurs experimentally in the absence of reinfection and is thus separate from the self-cure phenomenon. A confusing number of immune effectors have been identified with this phenomenon in various model systems, and likely some if not all are at some time active in the equine intestine. The mechanisms involved are T cell dependent. Antibodies may be involved but are not sufficient in themselves to induce expulsion. T cell–mediated mastocytosis, eosinophilia, and goblet cell hyperplasia have been demonstrated to be related to expression of expulsion in some systems. These accessory cells are involved in the nonspecific efferent arm of this response. Mediators of inflammation, such as vasoactive amines, prostaglandins, and increased mucus production, have been linked to immune elimination of primary infections in some but not all model systems. A number of specific immunologic events likely initiate several nonspecific effector mechanisms, resulting in this expulsion. These mechanisms vary with the species of parasite involved. The elimination of adult *S. westeri* and *P. equorum* from maturing horses and the hypothetical seasonal turnover in *Strongylus* spp. and cyathostome species may be mediated by such responses.

In addition to immune responses that occur during tissue migrations, protective resistance to reinfection by gastrointestinal nematodes occurs at the surface of the epithelium. This reaction, termed *rapid expulsion* or *immune exclusion,* is separate from self-cure or immune expulsion of primary infection. Infective larvae are expelled from the intestine in a matter of hours. Again mechanisms of expulsion described vary between parasite and host species. However, anaphylactic reactions and mucus entrapment have been observed. Some experiments using the *Trichinella spiralis*–rat system suggest that alterations in the epithelial cells in immune animals are involved

directly in the exclusion of these parasites. Although immune-mediated damage of intestinal helminths such as decreased fecundity, reduced size, and morphologic alterations have been noted, infective larvae expelled by rapid expulsion mechanisms remain viable and undamaged. One may speculate that reactions of this nature are responsible in part for resistance to reinfection of equines with cyathostomes.

TISSUE-MIGRATING PARASITES

A number of intestinal helminths migrate through extraintestinal tissues as part of their life cycle. These include parasites such as *P. equorum, Strongyloides westeri,* and *Strongylus* spp., all of which stimulate an acquired immune response in the horse. During this migration, larvae are vulnerable to attack by immune effectors that may encapsulate them in an immune-mediated inflammatory response, disrupt their migrations by interfering with important metabolic or invasive processes, or inhibit molting from the L_3 to L_4 stages. The most studied phenomenon in this regard is antibody-mediated adherence of inflammatory cells, which may result in killing of the larvae. This phenomenon has been demonstrated to involve many cell types and immunoglobulin isotypes in different host-parasite systems. In vitro studies of this nature have been conducted using *S. vulgaris* third-stage larvae and equine immune effectors in the author's laboratory. In these experiments, an antibody-dependent adherence of cells was demonstrated and shown to be parasite species-specific. In vitro killing was mediated by eosinophils and not by neutrophils or monocytes. Activated eosinophils were necessary to mediate this response, and *S. vulgaris* infections have been demonstrated to activate eosinophils and neutrophils in vivo.[26] Although eosinophils are not known with certainty to be essential in this protective immune response, an anamnestic eosinophilia is characteristic in immune ponies but not nonimmune ponies following experimental *S. vulgaris* challenge. Because of its prominence and compelling in vitro and correlative in vivo data, the eosinophil has been considered to be a major effector in immune-mediated helminth killing. However, recent studies in murine parasite model systems in which eosinophilia was blocked by anti–interleukin-5 treatment suggest that this type of cell is not essential for protective resistance in some systems.[27] Possibly, in vivo a number of cells function as effectors and may overcome the absence of sufficient eosinophils under some circumstances. Antibody reactivity with parasite-secreted enzymes and molting fluids, factors important in parasite homeostasis, have been demonstrated in vitro; and similar reactions may be important in vivo.

T cell responses are essential for the induction of protective resistance to tissue-migrating helminths in most systems studied, including the experimental *S. vulgaris* pony model. This dependency likely is caused by the T cell dependency of the antibody response and by the mediation of secondary effector cell responses. Antigenic substances secreted or excreted by migrating nematodes likely are important in the induction of these responses. A combination of immune responses elicited by a combination of specific parasite antigens, including surface antigens and secreted or excreted products, probably is necessary to induce an immune response sufficient to provide protective resistance.

Parasite Evasion of Immune Effectors

Some parasites that live for long periods within a host modulate the host response in a specific and a nonspecific fashion, as described previously for filarial infections. What is inferred is that this modulation inhibits immune responses associated with protective resistance. In addition, blocking antibodies that inhibit antibody-dependent eosinophil killing of schistosome larvae have been described in rodent models and in sera from infected patients. The role of these types of antibodies in other infections is unclear.

A number of parasite-driven mechanisms have been described that promote parasite survival within hosts possessing strong immune responses directed toward their antigenic components.[28] Others mask themselves to avoid recognition or directly inactivate immune effectors. Host molecules shown to be attached to the surface of parasites include glycolipids of blood group antigens, glycoproteins, serum proteins, and class I and II major histocompatibility complex molecules. In some instances, hostlike molecules have been demonstrated to be encoded in parasitic helminth genomes and expressed on their surfaces. This phenomenon, termed *molecular mimicry,* has been postulated to be a mechanism evolved by the parasite to mask itself from the host immune surveillance systems.

Some parasites, notably tapeworm larvae, produce factors that activate complement and may produce a state of localized complement depletion in vivo. Other factors from tapeworm larvae disrupt the coagulation cascade and others have antiinflammatory activity.[29] Filarial nematodes synthesize prostaglandins from host arachidonic acid and release these in vitro and potentially in vivo.[30] These molecules may be responsible for local modulation of inflammatory cell function and may play a role in the survival of parasites.

These types of mechanisms have been identified in most host-parasite systems examined and are likely active in the horse. These intriguing phenomena, however, have yet to be investigated in equine parasite infections.

Mechanisms of Anthelminthic Resistance

Three main target sites for anthelmintic activity against helminth infections are ion channels, microtubules, and energy-requiring transporters.[31] The tetrahydropyrimidines (pyrantal), imidazathiazoles (levamisole), macrocytic lactones (ivermectin and moxidectin), and piperazines target some type of ion channel. Microtubules are the main targets of benzimidazoles.[32] Salicylanilides and chlorinated sulfonamides target energy-requiring processes of the helminths.

Like bacterial resistance to antimicrobials, resistance to anthelmintics is associated with a genetic modification that transforms a susceptible population of parasites into a resistant one.[31] Intensive use and inappropriate or ineffective dosing of therapeutics contributes to development of resistance. Presumably, treatment eventually eliminates susceptible individuals, allowing for accumulation of resistance genes within the remaining population. Rather than passing genetically encoded material by transmissible elements, resistance is passed through successive generations of allelic inheritance.

Benzimidazoles are a useful class of anthelmintic because of their specificity for nonmammalian microtubules.[31] Interference in microtubule function disrupts the transport of vesicles that contain gut secretions to the outer surface of the parasite.[33] Resistance is thought to be due to selection of certain β-tubule allelic isotypes that are associated with the loss of high-affinity binding sites on the β–tubulin protein.[34] Specifically, the loss of the β-tubulin type 2 isotype allele from the population and the addition of a point mutation of the type 1 isotype results in attainment of high-level resistance in that population.[31,34]

Attainment of resistance to avermectins is complex and a major problem. Eventual resistance to milbemycins does occur. This phenomenon has been studied most extensively in *Haemonchus contortus* infections and *Caenorhabditis elegans*.[35] Macrocyclic lactones induce a flaccid paralysis that is mediated by interfering with the action of the glutamate-gated chloride channel. Acting as an agonist of glutamate, these anthelmintics prolong the opening of the channel. Three mutations of the genes encoding the glutamate-gated chloride channel are associated with high-evel resistance, and one to two changes result in some resistance.[36,37] Genetic changes in another set of nematode molecules, the P-glycoproteins also may be important in attainment of resistance.[31]

Pyrantel compounds are essentially acetylcholine agonists.[31] Spastic paralysis results from binding of the compound to the nicotinic acetylcholine receptor. Populations of worms appear to be heterogeneous in development of a spastic response to acetylcholine agonists. Resistance appears to occur from a shift to a homogenous population that is insensitive to the effect on the acetylcholine receptor.[38] Most of the testing has been performed with levamisole, but it appears that less sensitive receptor subtypes are also responsible for resistance to other nicotinic agents including pyrantel.[31]

Concern for the development of resistance in equine gastrointestinal parasites primarily centers on the development of resistance in cyathostomes.[32,39-45] Cyathostome resistance is a common finding for fenbendazole.[46] Fenbendazole resistance is apparent on 79% to 90% of farms tested in studies that use fecal egg counts as an indication of effectiveness of anthelmintic therapy. Even when sensitive, fecal egg counts are suppressed for less than 2 to 3 weeks. This resistance usually involves multiple species of small strongyles. Resistance to pyrantel is increasing. Efficacy has been reduced to as low as 60% effectiveness. Up to 30% of farms tested have demonstrated resistance to pyrantel. Dual resistance to fenbendazole and pyrantel has been demonstrated. Ivermectin resistance has not been reported for cyathostomes. Ivermectin, although still efficacious, suppresses egg counts for considerably less time than moxidectin.

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2.4—Rickettsial Diseases

Yasuko Rikihisa

Potomac horse fever (PHF) and equine ehrlichiosis are two rickettsial diseases known to affect horses. Diagnosis of these diseases requires an awareness of the typical pattern of clinical signs, hematologic values, seasonal pattern, and prevalence of the disease in the area. Because of the frequent international and domestic transport of horses, knowledge of the previous location of the horse also may aid in diagnosis. PHF is transmitted by oral ingestion of trematodes present in aquatic insects. Equine

ehrlichiosis is transmitted by ticks. For both diseases, serologic testing by indirect fluorescent antibody (IFA) test is the primary method of laboratory diagnosis. Polymerase chain reaction (PCR) methods are also useful for detection of both infections. When the diseases are diagnosed correctly at acute stages of infection and are treated properly, the prognosis for horses with equine ehrlichiosis is excellent and that for horses with PHF is excellent to good in uncomplicated cases.

Potomac Horse Fever

GEOGRAPHIC DISTRIBUTION

PHF (equine monocytic ehrlichiosis, equine ehrlichial colitis, or acute equine diarrhea syndrome), a rickettsial disease of equidae, was recognized originally in 1979 along the Potomac River in Montgomery County, Maryland.[1] The disease also may have existed in northern California since the 1970s.[2] PHF is now known to occur serologically in 43 of the United States, two provinces (Ontario and Saskatchewan) in Canada, France, Italy, Venezuela, India, and Australia. However, confirmation by isolation of the causative agent has been made only in the United States. The disease is caused by a rickettsial organism currently designated *Neorickettsia risticii* (formerly *Ehrlichia risticii*[3,4]), which is serologically distinguishable from other pathogenic species infecting horses.

CAUSE

In 1983, PHF was shown to be transmitted between horses by whole blood transfusion.[1] This finding made laboratory experiments under controlled conditions possible. In 1984, serologic cross-reactivity of sera of infected horses with *Neorickettsia sennetsu* (formerly *Ehrlichia sennetsu*), a human Sennetsu fever agent in Japan, was noted.[5] In the same year, the presence of ehrlichial organisms in macrophages and glandular epithelial cells of the intestinal wall of affected horses was demonstrated by transmission electron microscopy.[6] The causative agent was isolated independently in different laboratories using human histiocytic lymphoma U-937 cells,[7,8] canine primary monocytes,[5] and murine P388D$_1$ cells.[9,10] The agent was confirmed to reproduce disease in horses and was reisolated from horses with experimentally induced disease.[5,8,9]

N. risticii currently is classified among three *Neorickettsia* species.[4] The genus *Neorickettsia* belongs to the family Anaplasmataceae along with the genera *Ehrlichia* and *Anaplasma,* which are obligatory intracellular bacteria with a tropism for hematopoietic cells, and the genus *Wolbachia,* which consists of intracellular symbionts of invertebrates. All these microorganisms belong to the α subdivision of the proteobacteria. None of the family Anaplasmataceae has been cultured outside of eukaryotic cells or in yolk sacs.[11] Success in culturing *N. risticii* in macrophage cell lines made possible the obtaining of the quantities of organisms needed for basic research at the molecular and cellular levels and for development of serologic tests and vaccines.

The family Anaplasmataceae is included in the order Rickettsiales along with the family Rickettsiaceae.[4] 16S ribosomal RNA gene sequence comparison currently is considered to be the most reliable method to determine phylogenetic relatedness among bacteria. Studies have shown relatedness (81.7%) in the sequences of the 16S rRNA gene in *N. risticii* and *Rickettsia prowazekii,* which is the causative agent of epidemic typhus, a well-known rickettsial disease responsible for the deaths of millions of persons during wartime and natural disasters. The percentage of 16S rRNA gene sequence homology between *N. risticii* and *N. sennetsu* is 98.9%, and the next related bacterium is *N. helminthoeca* (94.8% 16S rRNA gene sequence homology).[12] *N. helminthoeca* is the causative agent of salmon poisoning disease of the dog. *N. helminthoeca* is transmitted by trematodes, is antigenically cross-reactive with *N. risticii,* and biologically and morphologically resembles *N. risticii.*[13] Furthermore, the 16S rRNA gene of the trematode *Stellantchasmus falcatus* (SF agent) is 99.1% homologous to that of *N. risticii.*[14] Within 11 *N. risticii* strains, a maximum of 10 nucleotides are different (0.7% divergence).[15]

N. risticii is genetically divergent from *Anaplasma phagocytophilum* (formerly *Ehrlichia equi*)—which causes equine ehrlichiosis, tick-borne fever, and human granulocytic ehrlichiosis (HGE)—and *E. canis,* which is the type species of the genus *Ehrlichia,* by 16S rRNA gene sequence comparison, homology being 83.3% and 82.4%, respectively.[16]

N. risticii is a tiny gram-negative coccus and stains dark blue to purple with Romanowsky's stain (Figure 2.4-1). *N. risticii* tends to occupy one side of the cytoplasm rather than being symmetrically or evenly distributed. *N. risticii* generally is round but sometimes is pleomorphic and may be elongated, especially in tissue culture. The organism divides by binary fission and is found in membrane-lined vacuoles within the cytoplasm of infected eukaryotic host cells, primarily macrophages and glandular epithelial cells in the intestine of the horse. *N. risticii* occurs in at least two different forms: multiple dark, small organisms (0.2 to 0.4 μm) enveloped by the host membrane (called morulae) and relatively light, large forms (0.8 to 1.5 μm) individually tightly wrapped with host membrane.[17,18] Morulae appear to interchange with individually enveloped forms, because an intermediate stage appearing as moderately dense ehrlichial cells tightly enveloped with the host membrane that is continuous with the membrane surrounding a morula,

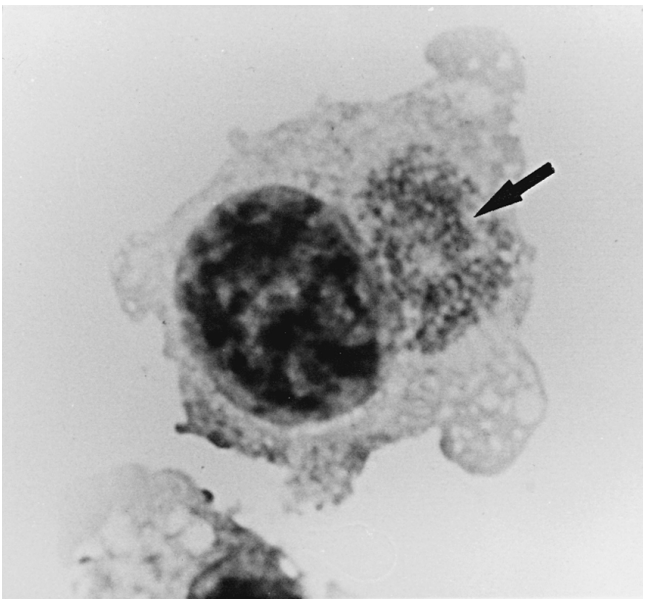

Figure 2.4-1 *Neorickettsia risticii* in the cytoplasm of $P388D_1$ cells. Organisms are stained dark purple of Diff-Quik staining *(arrow)*. (×4000.)

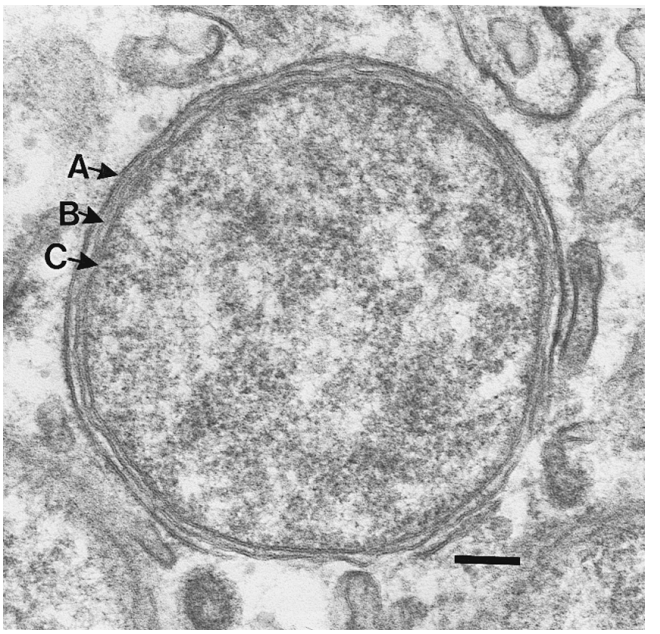

Figure 2.4-2 Transmission electron micrograph of *Neorickettsia risticii* in the cytoplasm of a macrophage in the large colon of a horse. *N. risticii* is enveloped tightly by the host membrane *(A)* and has its own outer *(B)* and inner *(C)* membranes. (×105,000.) Bar = 0.1 μm.

(Reproduced with permission of Yasuko R: Ultrastructure of rickettsiae with special emphasis on ehrlichiae. In Williams JE, Kakoma I, editors: *Ehrlichiosis,* Dordrecht, The Netherlands, 1990, Kluwer Academic Press. Reprinted by permission of Kluwer Academic Publishers.)

has been seen.[19] *N. risticii* in T-84, $P388D_1$, and U-937 cells, in primary equine monocyte culture, and in infected equine tissues is seen primarily in individual forms, especially in intestinal epithelial cells.[19,20] However, several recent *N. risticii* isolates make inclusions as large and as tightly packed as *E. canis* inclusions.[21,22] Vacuoles containing *N. risticii* do not fuse with lysosomes. The inhibition of lysosomal fusion is not a generalized process but rather is restricted to vesicles that contain *N. risticii*.[23]

Transmission electron microscopy reveals that *N. risticii* has distinct ribosomes and DNA strands.[17-19] Clumps of ribosomes are distributed homogenously in the cytoplasm rather than being marginated beneath the cytoplasmic membrane. Compared with those of the rickettsiae or ordinary bacteria, the DNA and ribosomes in *N. risticii* are packed more loosely in the cytoplasm except for the small, dense form. Thus the electron density of ehrlichial organisms is similar to that of the host cell cytoplasmic background, and this low contrast makes finding *N. risticii* in infected tissue difficult at low magnification under the electron microscope. *N. risticii* is surrounded by thin, bileaflet outer and inner membranes (Figure 2.4-2). Unlike the rickettsiae, ehrlichial organisms show no thickening of either leaflet of the outer membrane.[18,19] Morphologically, *N. risticii* does not appear to contain significant amounts of peptidoglycan.[19] Peptidoglycan is counterproductive when considering the intracellular life of *N. risticii* because the organism requires the provision of an effective diffusion rather than mechanical protection by peptidoglycan.

No plasmid or phage has been detected by agarose gel electrophoresis of DNA fractions from *N. risticii* cultured in vitro. Lipopolysaccharide is not readily detectable by the conventional extraction method or silver staining.[11] Recent analysis of *N. sennetsu* and *A. phagocytophilum Ehrlichia chaffeensis* genome sequencing data confirms the absence of genes for lipid A biosynthesis and most of genes for peptidoglycan biosynthesis.[23a] No endospore-like structure has been reported, although minicell-like structures resulting from uneven binary fission and apparent outer membrane vesicles of various sizes have been seen in *N. risticii*.[18]

N. risticii appears to perform aerobic and asaccharolytic catabolism. The metabolic activities of *N. risticii* have been investigated. *N. risticii* can use glutamine and glutamate and generate adenosine triphosphate as rickettsiae do.[10,24] The preference of glutamine over glutamate is likely because glutamine penetrates phagosomes better than glutamate. *N. risticii* cannot use glucose-6-phosphate or glucose[10] and is similar to the genus *Rickettsia*, but the rate of adenosine triphosphate synthesis is much lower than in *Rickettsia typhi*.[24] The greatest metabolic activity of *N. risticii* is observed at a pH of 7.2 to 8.0 and declines drastically at a pH below 7.[10]

This finding suggests that *N. risticii* can prevent acidification and lysosomal fusion of the vacuole where it resides.

EPIZOOTIOLOGY

No sex, age, or breed predilection exists for equine infections based on serologic testing of horses in Ohio.[25] The disease occurs along big rivers and their tributaries mainly during the summer and is most common from June through September. DNA from *N. risticii* has been detected in virgulate cercariae in freshwater operculate snails (Pleuroceridae: *Juga yrekaensis*) from northern California,[26,27] in virgulate xiphidiocercariae isolated from snails (Pleuroceridae: *Elimia livescens*) in central Ohio,[28] and from snails (Pleuroceridae: *Elimia virginica*) in central Pennsylvania.[29] This type of trematode is known to become metacercaria in aquatic insects, and *N. risticii* DNA has been detected in mesocercaria and metacercaria in various aquatic larval and adult insects such as mayflies and caddisflies in northern California[30] and in central Pennsylvania.[29] One horse fed adult caddisflies in northern California[31] and two horses fed adult caddisflies or a mixture of adult caddisflies and mayflies in central Pennsylvania developed PHF.[29] 16S rRNA gene sequences and 51-kd antigen amino acid sequences obtained from culture isolates from horses, horse blood, and caddisflies were identical, proving oral transmission and establishment of stable infection of *N. risticii* in the horse as the cause of PHF in Pennsylvania.[29] Ingestion of the larval stage of aquatic insects or cercariae in the water did not transmit *N. risticii*.[31] Blood-sucking arthropods such as *Dermacentor variabilis,* black flies, eye gnats, biting midges, and mosquitoes have been found to be negative for *N. risticii* transmission.[32]

Experimental injection of cell-cultured *N. risticii* or infected horse blood and reisolation of the organism have revealed that dogs, cats, mice, and nonhuman primates can be infected, although only mice and cats developed significant clinical signs.[33-36] PHF may have zoonotic potential, because *N. risticii* can cause mild clinical signs in primates.[36] Seroepizootiologic studies showed that cats, dogs, foxes, pigs, and goats in an endemic area of Maryland develop antibodies to *N. risticii*.[37] The reservoir potential of these animals is currently unknown. Dogs demonstrating symptoms of canine ehrlichiosis were seropositive to *N. risticii* (but not *E. canis*), and an agent identical to the 16S rRNA *N. risticii* was isolated from the dogs in this report.[38] However, the potential for this canine isolate to infect horses and cause PHF is undetermined.

Owners and veterinarians should be aware of the sporadic nature of PHF. On some farms and racetracks, the disease recurs several summers in a row. Alternatively, the disease may appear suddenly in isolated areas where no previous PHF cases have been reported. Usually, if clinical cases are confirmed serologically on a farm, racetrack, or horseshow, several additional seropositive horses show no clinical signs. Of horses at two racetracks in Ohio, 13% (138 of 840) to 20% (116 of 574) were exposed to *N. risticii,* according to the results of a serosurvey conducted in the summer of 1986.[25] Of seropositive horses, 80% to 90% did not show clinical signs of infection.

PATHOGENESIS AND IMMUNE RESPONSES

Establishment of Infection

N. risticii can establish infection in mice and horses with or without causing apparent clinical illness. In a murine model of PHF, disease is apparently dose dependent.[39] Only at higher dosages can the organism cause disease and pathologic changes. In mice, susceptibility varies among strains.[40]

N. risticii can be reisolated from the peripheral blood monocytes of horses starting from day 6 to day 11 after ingestion of adult aquatic insects harboring *N. risticii,*[29] and ehrlichemia persists 1 to 2 weeks after spontaneous resolution of clinical signs.[29,41,42] *N. risticii* may persist much longer in the intestinal walls of clinically recovered horses, for the homogenate of intestinal tissues recovered from two ponies at 2 months after infection contained *N. risticii* antigens.[43] Furthermore, in a pregnant mare-fetus system, *N. risticii* infection persists up to 4 months, for *N. risticii* was isolated from the spleen, bone marrow, and mesenteric and colonic lymph nodes of a 7-month-old fetus of a mare that was infected experimentally during the third month of gestation.[44]

Mechanism of Diarrhea

N. risticii infects blood monocytes and has a predilection for the intestinal wall, especially that of the large colon where it infects tissue macrophages,[6,17] intestinal glandular (crypt) epithelial cells, and mast cells.[16,17] *N. risticii*–infected cells are shed into the intestinal lumen as detected by electron microscopy[18] and by PCR of fecal specimens.[31,42] Watery diarrhea is caused by a reduction in electrolyte transport (Na^+, Cl^-); thus a lack of water resorption occurs, mainly in the large and small colons.[45] Infected intestinal epithelial cells lose microvilli,[20,45] which may contribute to the reduced electrolyte transport and water resorption. An increase in intracellular cyclic adenosine monophosphate is found in infected mouse macrophages and infected mouse and horse intestinal tissues.[45-47] This change in cyclic adenosine monophosphate content also may contribute to the reduced lumenal absorption of Na^+ and Cl^- in the colon and thus to the lack of water absorption and diarrhea.

Cellular Damage and Cytokines

In contrast to cells infected with virulent strains of the genus *Rickettsia,* host cells infected with *N. risticii* show little cytolysis in vivo or in vitro until the cytoplasm is filled completely with infecting organisms and cells

burst. Ehrlichial release appears to occur not only by cell lysis but also through exocytosis by fusion of the inclusion membrane with the plasma membrane. In the case of intestinal epithelial cells, which make a monolayer tightly connected by circumferential zones of intercellular junctions, ehrlichial organisms appear to be transmitted between adjacent cells in vitro by a coupled exocytosis in one cell and endocytosis in an adjacent cell.[20]

Unlike ordinary gram-negative bacteria, *N. risticii* does not activate macrophages fully in vitro. *N. risticii* induces only low levels of tumor necrosis factor α and prostaglandin E_2 production in macrophages. In contrast, infected macrophages produce large amounts of interleukin-1, and this may be related to the pathogenesis of the disease.[46] This difference may be caused by a lack of lipopolysaccharides in *N. risticii*. *N. risticii* in general does not induce a severe inflammatory reaction in equine tissues.[18,45]

Mechanism of Infection of Macrophages

Because *N. risticii* is an obligatory intracellular bacterium, the organism must bind to the proper receptor to induce internalization and to maintain an intracellular environment adequate for survival and proliferation. In vitro, professional phagocytes (monocytes and macrophages) of host animal species have no intrinsic resistance to infection with *N. risticii*. In addition, intestinal epithelial cells and mast cells, which are normally not phagocytic, are induced to take up *N. risticii*. *N. risticii* binds to equine macrophages but not polymorphonuclear leukocytes. *N. risticii* entry into $P388D_1$ cells is blocked by monodansylcadaverine, an inhibitor of transglutaminase that inhibits receptor-mediated endocytosis, suggesting entry in this fashion.[48] Ehrlichial internalization, proliferation, and intercellular spreading, but not binding, depended highly on the host transglutaminase and microtubule system,[48,49] as well as on calcium-calmodulin[50] and protein tyrosine kinase.[51]

N. risticii can be taken up readily by equine polymorphonuclear leukocytes but is destroyed rapidly in those cells.[48] Polyclonal equine antiserum to *N. risticii* does not inhibit binding or internalization of *N. risticii* to $P388D_1$ macrophages, but internalized antibody-coated *N. risticii* fails to survive.[52] Because the antigen-binding fragment of the equine anti–*N. risticii* immunoglobulin G (IgG) blocks the binding of *N. risticii* to $P388D_1$ macrophages, antibody-coated *N. risticii* most likely enters macrophages via the Fc receptor. The mechanisms of destruction of antibody-coated *N. risticii* in macrophages are presently unknown.

Immune Response

Recovered horses have been reported to be resistant to development of clinical disease on rechallenge for at least 20 months.[53] Humoral and cell-mediated immune responses appear to have significant roles in this protection. *N. risticii* induces a specific antibody response in the natural host or in experimental animals, regardless of the presence of clinical signs. The presence of the antibody, however, does not always correlate with clearance of ehrlichial organisms and presence of protective immunity. An IgM response occurs a few days after the initial infection and lasts for less than 2 months. A challenge injection did not elicit an IgM response. Several days after *N. risticii* infection of the horse, tests for IgG antibody became significantly positive.[54]

Development of neutralizing antibodies in infected horses also was demonstrated using a cell culture system and a murine model for PHF.[41] Three mechanisms of antibody neutralization have been shown. In the first mechanism, antibody blocks ehrlichial binding to its specific receptor, instead making it bind through the Fc receptor. In the second mechanism, the antibody directly inhibits ehrlichial metabolism as demonstrated by reduced $^{14}C\text{-}CO_2$ production from ^{14}C-L-glutamine in host cell–free *N. risticii* in the presence of the antibody.[52] The third mechanism is antibody-dependent cell-mediated cytotoxicity. Macrophages infected with *N. risticii* present ehrlichial antigens on their surface similar to virus-infected cells and are lysed when incubated with antiehrlichial antibody and normal equine peripheral blood monocytes.[55] Five to seven major antigens were exposed on the surface.[56] These antigens are considered to be the target in antibody-mediated neutralization.

Although mouse resident peritoneal macrophages fail to generate superoxide on interaction with *N. risticii*, peritoneal macrophages from mice previously inoculated with *N. risticii* and further sensitized with *N. risticii* antigen inhibit *N. risticii* in vitro by generating superoxide.[57,58] Interferon-γ has the ability to activate uninfected and infected macrophages and transform them into effector cells. The ehrlichiacidal mechanism exhibited by interferon-γ was investigated using murine peritoneal macrophages. *N. risticii* was highly sensitive to nitric oxide, which was generated by macrophage cytoplasmic nitric oxide synthase induced by interferon-γ.[59] Spleen cells from recovered mice at 28 days after inoculation with *N. risticii* proliferated in response to *N. risticii* antigen.[60] Activities of T cells that generate interferon-γ are, however, severely depressed in infected mice in a dose-related manner.[39] One cause for depressed T cell response was found in macrophages. Class II histocompatibility antigen induction on the surface of *N. risticii*–infected macrophages (antigen-presenting cells) is suppressed in vitro,[61] suggesting inhibition of antigen-specific T cell activation in *N. risticii* infection. Thus an understanding of the immunodepression mechanism and

identification of T cell stimulating and ehrlichial antigens that induce neutralization are keys toward developing an improved, more effective vaccine for PHF.

CLINICAL FINDINGS

The incubation period for *N. risticii* infection is approximately 1 to 3 weeks. Clinical signs are an acute onset of fever up to 107° F, depression, anorexia, decreased borborygmi in all abdominal quadrants, subcutaneous edema of the legs and ventral abdomen, dehydration, and diarrhea. Laminitis and severe abdominal pain occur in 15% to 25% and 5% to 10% of cases, respectively, which are the major reasons for euthanasia.[62] Laminitis may progress, despite resolution of other clinical signs. Diarrhea may be mild to severe "pipestream" and occurs in 10% to 30% of cases. In some horses diarrhea may be transient; in others cases diarrhea persists for several days; and still other horses may have no diarrhea. Owners and veterinarians should be aware of the variable nature of clinical signs. Case fatality rates vary from 5% to 30%. Transplacental transmission of *N. risticii* is reported, and the organism may induce abortion or resorption of the fetus or produce maladjusted foals, which require extensive neonatal care.[44,63-65] Recurrence of diarrhea and on and off prolonged illness in antibiotic-treated horses has been observed.[66] Leukopenia (white blood cell count <5000/μL) with a left shift and rebound leukocytosis (white blood cell count >14,000/μL) are prominent hematologic changes. Anemia, changes in plasma protein concentration, increased packed cell volume, and thrombocytopenia also may be observed.[67]

DIAGNOSIS

In contrast with *A. phagocytophilum* infection, visual observation of blood smears of suspected horses after Romanowsky staining is useless for diagnosis of *N. risticii* infection, because only a few blood monocytes are infected with a few organisms even at the acute stage of infection.

Serologic Diagnosis

All ehrlichial agents induce specific humoral immune responses that are the basis for serologic diagnosis of ehrlichial diseases. However, because serologic response occurs in every animal exposed to *N. risticii,* regardless of whether infection is established or disease is present, serologic testing alone, with no supporting information, provides limited information for the diagnosis of disease. Serologic diagnosis of ehrlichial infections is performed primarily by IFA.[54,68] The cutoff titer for positive serologic result may vary with the laboratory. In the author's laboratory, IgG IFA titers of 20 or higher represent a positive serologic result. For serologic testing for *N. risticii* infection, the higher the titer is, the greater the correlation is with PHF clinical disease. The chance of healthy horses having a titer of 40 or less is similar to that of ill horses (early and late stages, and low levels of infection with *N. risticii* or possible cross-reaction with other microorganisms). Thus at a titer of 40 or less, although the horse is seropositive, the current disease is not likely to be caused by *N. risticii*. The chance of having a titer of 80 or more and 640 or less is approximately 4 times higher in ill horses than in healthy horses, and the chance of having titers higher than 1280 is 12 to 26 times higher in ill horses.[25] In addition, all experimentally infected horses had an IFA titer of 80 or higher at the onset of clinical disease.

N. risticii has been adapted to grow in a continuous murine monocyte-macrophage cell line and other cell lines; thus antigen slides are easy to prepare.[54,69] IFA testing may produce false-positive results with equine sera when they bind nonspecifically to infected cells. Several means of solving this problem are these: First, *N. risticii* organisms should be seen clearly in the cytoplasm of the host cells by IFA staining at magnification of 1000 times. If staining is blurred, morphologically different from positive control staining or Giemsa-stained infected cells or if extracellular objects are stained, the sample should not be judged positive. Second, positive and negative control equine sera must be tested to ensure the quality of the entire IFA procedure each time, including the quality of the antigen slides used and fluorescein isothiocyanate anti-horse IgG. Third, to reduce nonspecific binding, the author's laboratory uses 0.02% Tween-20 in the phosphate buffered saline washes. Usually serial dilution of the serum takes care of the problem of nonspecific binding if the titer of the sera is sufficiently high. If none of these procedures work, the author preabsorbs the equine sera with uninfected macrophages cultured and prepared under the same condition as the *N. risticii*–infected macrophage antigen. Western immunoblot analysis has been developed and is useful in distinguishing sera that have a nonspecific IFA reactivity or react by IFA to cross-reacting antigens.[70,71]

An enzyme-linked immunosorbent assay (ELISA) has been developed using purified *N. risticii* as the antigen.[54] The IgM titer rises earlier during the course of infection than the IgG titer. A rise in IgM titer occurs only at the time of initial infection, and the titer becomes negative in 1 to 2 months.[55] Thus an IgM ELISA is not useful for detecting chronic or multiple infections but is useful for early diagnosis of a primary infection of nonvaccinated horses. Several other serologic tests such as latex agglutination[72] and monoclonal antibody–based competitive ELISA have been reported.[73] However, to date a rapid and reliable field serodiagnostic test is not yet available.

No significant serologic cross-reactivity occurs between the family Anaplasmataceae and the families Rickettsiaceae

and Chlamydiaceae by the IFA test.[3,5,11] *N. risticii* has only minor heat-sensitive antigenic determinants, unlike the genus *Rickettsia*. By Western blot (immunoblot) analysis, however, cross-reactivity of 55-kd heat-shock protein 60 homolog with those of other *Neorickettsia, Anaplasma,* and *Ehrlichia* spp. and *Rickettsia* spp. is detected.[74,75]

Several ehrlichial species antigenically cross-react within the genus when tested by IFA or Western blot analysis.[13] Within the genus *Neoricketsia, N. risticii* and *N. sennetsu* share the strongest common antigens. *N. sennetsu* is nonpathogenic to horses but it protects them from *N. risticii* infection.[71]

Immunologic cross-reactivity among members of the genus *Neorickettsia* does not create a serious problem in serologic diagnosis because of the host animal specificity of *Neorickettsia* spp. in nature and different clinical signs. However, because homologous species or strain antigen provide the most sensitive serodiagnosis, identifying at least the species is still important. Even if infection is misdiagnosed with other species, tetracycline series of antibiotics are effective for all of the ehrlichial diseases, especially at early stages of infection.

Recently the author's laboratory found that one of six *N. risticii* Ohio isolates and three Kentucky isolates were divergent from *N. risticii,* type strain (ATCC VR-986), in their antigen profiles by Western immunoblot analysis and by IFA using two panels of monoclonal antibodies.[21] The isolates also are different in the base sequence of 16S rRNA[15] and translated amino acid sequences of 51-kd antigen genes[28] and in growth morphology in the cell.[22] The 51-kd protein is a unique antigenic protein cloned from the *N. risticii* genome that shows no homology to any other known protein.[76] Amino acid sequences of 51-kd protein derived from *Juga* sp. snails in California differ at 17 amino acid positions over the 179 amino acids that can be compared (90.5% identity) with those of caddisflies and horses infected by ingestion of the caddisflies in Pennsylvania. However, the 51-kd protein amino acid sequence from caddisflies in Pennsylvania differs only at 7 amino acid positions (98.6% identity) from *N. risticii* Maryland strain over the 483 amino acids that can be compared.[29] An association of heterogeneity of *N. risticii* strains and PHF vaccine failure in Virginia and Maryland was reported.[77] Thus multiple strains or subspecies of *N. risticii,* or *Neorickettsia* spp. in the field, are likely to be distinguishable by Western immunoblotting or monoclonal antibody labeling of antigens but not by IFA or ELISA serologic testing of infected horse sera. Sequencing the antigenic protein genes further clarifies these strains. The existence of divergent antigenic variants should be taken into consideration in improving the serodiagnosis and efficacy of vaccines for PHF in the field.

In summary, recommendations for the serologic confirmation of PHF include demonstration of seroconversion, a fourfold rise or fall in titer, or the presence of IgM antibody specific to *N. risticii*. One should emphasize, however, that the initial diagnosis and treatment of PHF should be made on clinical signs, negative test results for other pathogens such as salmonella, for which the use of tetracyclines may be contraindicated, and initial IFA test results if available within 1 to 2 days. Treatment should not be delayed until laboratory confirmation is obtained from a second IFA test. It is important to point out that in the field PHF and salmonellosis can occur concurrently.[70] Weak seropositive or seronegative results of vaccinated horses when they develop clinical signs compatible with PHF are useful for ruling out the possibility of this infection. Seropositive results in vaccinated horses are useless unless confirmed by a fourfold rise in titer or by a positive PCR test.

Isolation of *Neorickettsia risticii* and Polymerase Chain Reaction

The isolation of *N. risticii* is accomplished by inoculating buffy coat or mononuclear cell fractions of peripheral blood of affected animals into cell culture.[21,22,42] Murine monocyte-macrophage P388D$_1$ cells, human histiocytic lymphoma U-937 cells, or canine primary monocytes are used for isolating *N. risticii*. The procedure is more sensitive than is direct observation. Even if a single organism cannot be seen in the original buffy coat smear, the organisms can be isolated by this procedure.

Isolation procedures for diagnosing infection, however, are impractical for clinical laboratories because they take too long (3 days to 1 month) relative to the rapid course of PHF and require a reasonable tissue culture technique and facility. Although positive isolation provides a definitive diagnosis, negative results do not necessarily indicate the absence of infection.

A PCR that detects 16S rRNA, *groel,* and 51-kd protein genes of *N. risticii* in peripheral blood monocytes and feces of experimentally infected animals has been developed.[26,42,78] Applicability of this test in field cases has been tested.[42] Although cell culture isolation gives superior sensitivity to PCR procedure for field specimens, PCR procedure will be used more often in the future because of its convenience.

Postmortem Identification of Ehrlichial Organisms

N. risticii can be demonstrated in intestinal epithelial cells and macrophages in paraffin-embedded tissue specimens with a silver stain or an immunoperoxidase procedure using a specific antibody to *N. risticii*. Immunoperoxide staining detects fewer *N. risticii* than

does silver staining, presumably because of partial inactivation of antigens owing to fixation and paraffin embedding. However, silver staining produces background reactions in some specimens and thus is less specific than immunoperoxidase staining.[79] These procedures have not been adapted for routine diagnosis.

PATHOLOGIC FINDINGS

The most obvious postmortem findings in horses with PHF are the grossly distended large colon and cecum filled with watery contents (Figure 2.4-3). Few other consistent gross pathologic changes in horses with PHF occur except for patchy hyperemia along the wall of the large intestine. No destruction, foul odor, or significant inflammatory infiltration occurs such as in enterocolitis caused by salmonella, which may cause similar clinical signs or may co-infect with *N. risticii* in nature.[45] However, remarkable goblet cell mucus depletion and reduced height and increased basophilia of mucosal epithelial cells, dilation of intestinal glands, and entrapment of cellular debris in the glandular lumens are seen in experimental *N. risticii* infection.[45,71,80] Mesenteric lymph nodes are small and consist of prominent sheets of histiocytes, macrophages, occasional giant cells, and severely depleted inactive lymphoid cells.[71] Lack of severe lesions and absence of neutrophil infiltration are thus important in the differential diagnosis of PHF. The combination of lymphohistiocytic enterocolitis, hepatitis, and myocarditis in the aborted fetus at approximately 7 months gestation because of *N. risticii* infection is reported.[65,66]

THERAPY

In vitro, *N. risticii* is susceptible to doxycycline, demeclocycline, and oxytetracycline but is resistant to erythromycin and nalidixic acid.[81] As with other rickettsiae, *N. risticii* is resistant to aminoglycosides because they penetrate host cells poorly. Although oxytetracycline alone is bacteriostatic, it was found to induce lysosomal fusion with ehrlichia-containing vacuoles in $P388D_1$ cells,[23] thus becoming bactericidal. Oxytetracycline and doxycycline frequently correct the pyrexia and other clinical signs of PHF within 24 to 48 hours, which by itself is diagnostic.[67] Although doxycycline is more effective than is oxytetracycline in vitro and is highly effective in mice inoculated with *N. risticii*,[81,82] oxytetracycline is preferred for the equine species because intravenous administration of doxycycline has an acute toxic effect.[83] Intravenous oxytetracycline at 6.6 mg/kg of body mass twice a day for 7 days was effective in 71% (five of seven) of experimentally infected ponies when ponies were treated after development of severe clinical signs.[67] Oxytetracycline is effective when given immediately after the development of fever but before development of diarrhea.[84] In the murine model of PHF, doxycycline, given on the fifth day, has the best effect on the immune response. When doxycycline is given on the third or seventh day after infection, immunodepression caused by *N. risticii* infection is more severe.[82] Although erythromycin or rifampin alone has a poor effect in controlling *N. risticii* infection in vitro or in infected mice,[80,81] the orally administered combination of erythromycin and rifampin has been efficacious in treating experimentally infected horses.[85]

Other supportive therapy frequently is required for acutely ill horses if they are to show clinical improvement. Dehydration is corrected by the oral or intravenous administration of polyionic isotonic fluids. Brief therapy (2 to 7 days) with nonsteroidal antiinflammatory agents (e.g., phenylbutazone, flunixin, meglumine, or aspirin) for horses may be valuable early in the course of treatment to help in the management of clinical signs of PHF.[84]

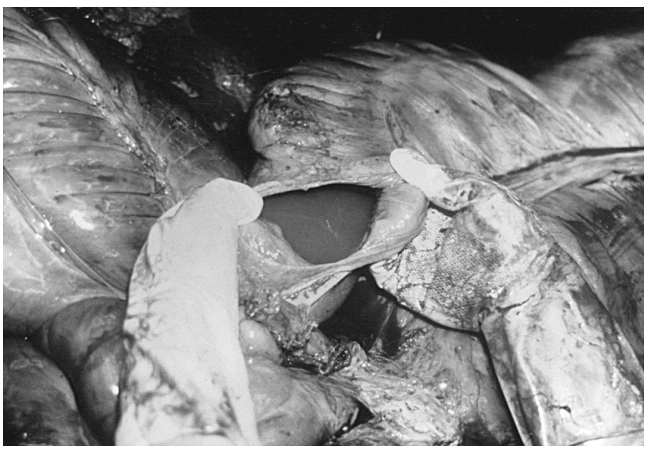

Figure 2.4-3 Fluid-filled large colon of a horse with Potomac horse fever.

PREVENTION

Ingestion of infected trematodes is probably the only means of transmission under natural circumstances, because susceptible animals housed for several months adjacent to infected animals do not have a higher incidence of the disease than those kept far from infected animals.[86] Because infection-induced immunity is excellent[53] and a vaccine made of β-propiolactone–inactivated host cell–free *N. risticii* protects mice from homologous challenge based on clinical, pathologic, and immunologic criteria,[87] vaccination is expected to be effective in controlling PHF. Vaccines prepared from inactivated cell-cultured *N. risticii* are available for PHF from three commercial sources. Formalin-inactivated whole organism vaccine with aluminum hydroxide adjuvant has been reviewed. The vaccine is reported to cause no ill effects

in horses except for swelling at the injection site in 10% of the animals.[88] Studies published in 1987 reported 78% prevention of all clinical signs except fever.[89] Protection conferred by this vaccine appears to be much shorter in duration compared with infection-induced protection. IFA titers measured after vaccination are also lower and drop rapidly in contrast to what occurs in natural infection.[88] Vaccination is not necessary for up to 2 years in the horse that has recovered from illness or has a high IFA titer from natural exposure or subclinical infection, because infection-induced immunity provides better protection than do vaccines. For nonexposed animals, considering the time required to develop immunity after vaccination, the short-lasting immunity to even the homologous strain of *N. risticii,* and the existence of antigenic variants in the field, how much benefit the vaccine will provide in the field challenge is unknown. Vaccine failures have been reported.[21,42,66,77] Therefore improvement of vaccine for PHF is desired.

Recombinant clones that produce several *N. risticii* antigenic proteins have been produced in the author's and other laboratories. Using the murine model for PHF, a combination of two recombinant clones that express 44-kd and 70-kd antigens was shown to confer protection against *N. risticii* challenge.[90] The protection was slightly less effective than the protection given by whole *N. risticii* antigen. The efficacy of recombinant antigens for protecting horses from PHF has not been reported.

Equine Ehrlichiosis

GEOGRAPHIC DISTRIBUTION

Equine ehrlichiosis and *Anaplasma phagocytophilum* in the cytoplasm of blood neutrophils and eosinophils in affected horses were reported initially in 1969.[91] The disease has been observed chiefly in California. In addition, sporadic cases have been reported in Colorado, Illinois, Florida, Washington, and New Jersey and also in Germany,[92] Switzerland,[93] Sweden,[94] and Israel.[95] In the United States the disease occurs much less frequently than does PHF.

CAUSE

A. phagocytophilum is classified along with *A. marginale,* which causes infectious anemia in cattle by infecting erythrocytes, and *A. platys,* which causes canine cyclic thrombocytopenia by infecting platelets, in the family Anaplasmataceae. Because 16S rRNA gene sequences differ only up to three bases among former *Ehrlichia equi; E. phagocytophila,* which infects ruminants (primarily sheep and goats in Europe); and recently discovered human granulocytic ehrlichiosis (HGE) agent, these organisms are now considered strains of *A. phagocytophilum*.[4] By 16S rRNA gene sequence comparison, relatedness between *N. risticii* and *A. phagocytophilum* is low, approximately 83.3%. *A. phagocytophilum* has been cultured in vitro using the tick-embryo cell line IDE8[96] and a human promyelocytic leukemia cell line HL60.[97] Contrary to previous speculation by some researchers, *A. phagocytophilum* is a distinct species different from *E. ewingii* (another granulocytic ehrlichia that infects dogs in the United States) and has a 16S rRNA gene sequence homology of 92.4%.

A. phagocytophilum appears as round, dark purple, small dots or loose aggregates that look like mulberries (morulae) in the cytoplasm of blood granulocytes, primarily in neutrophils and eosinophils by Romanowsky staining.[91] By electron microscopy, several loosely packed ovoid, round, or rod-shaped *A. phagocytophilum* organisms are seen in several membrane-lined vacuoles of equine neutrophils and eosinophils.[98] The size of vacuoles ranges from 1.5 to 5 μm in diameter.[99]

EPIZOOTIOLOGY

In contrast to PHF, equine ehrlichiosis occurs during late fall, winter, and spring. Like PHF, in some areas equine ehrlichiosis is endemic and in others the disease is absent. In one seroepidemiologic survey, horses residing in the foothills of northern California had greater exposure to *A. phagocytophilum* than did those residing in the Sacramento Valley.[100] Because most seropositive horses in this study were healthy, subclinical infection with *A. phagocytophilum* seems to occur as PHF. Recent findings indicate that the western black-legged tick, *Ixodes pacificus,* is able to transmit *A. phagocytophilum* and may act as a competent vector in the field.[101,102] *A. phagocytophilum* can cause parasitemia in dogs, cats, goats, sheep, and nonhuman primates with mild to no clinical signs.[91,103,104] Cattle, rats, mice, guinea pigs, hamsters, or rabbits are not susceptible to infection.[103,104] Gribble suggested that the horse may be an accidental host, based on the low incidence of the disease.[91] Whether other species of animals can serve as a reservoir for equine ehrlichiosis is unknown, although white-footed mice recently were shown to serve as an enzootic reservoir of the HGE agent.[105]

A structural protein of *A. phagocytophilum* called *ank* has repeats in amino acids that are similar to the repeats within the human erythrocyte ankyrin protein. Comparison of these amino acid sequences in *A. phagocytophilum* isolated from horses, a dog, cattle, ticks, and human patients from several geographic regions in the United States and Europe revealed that the isolates are heterogeneous and not segregated by host animal species, but rather segregated by geographic locations.[106,107] Intravenous inoculation of horses with infected human blood produces a disease indistinguishable from that caused by *A. phagocytophilum,*[108] and like *N. risticii,* diverse gene

sequences detected among horse isolates indicate equine ehrlichiosis is caused by multiple strains of organisms.

PATHOGENESIS AND IMMUNE RESPONSE

Infected animals develop antibody detectable by IFA, and leukocytes from infected animals show inhibition of migration when mixed with *A. phagocytophilum* antigen. Because *A. phagocytophilum* from the horse has not been propagated in vitro in sufficient quantity, an antigen prepared from the buffy coat cells of an experimentally infected horse is used as the antigen for immunologic studies.[109] Spontaneously recovered animals are immune to reinfection from 2.5 to 20 months. Maternal antibody protects foals from the disease for up to 2 months, but not from establishment of infection. When an immune mare was challenged, small numbers of infected neutrophils were seen in a 15-day-old foal born to the mare.[91] Oxytetracycline treatment eliminates *A. phagocytophilum* from horses, but rechallenge induces minimal clinical signs, suggesting the development of protective immunity in the recovered horse. Although the blood of recovered horses is reported not to be infectious, blood collected from ponies at 81 or 114 days after primary infection with *A. phagocytophilum* induced mild clinical signs (fever and mild thrombocytopenia) in susceptible recipient ponies but did not protect the recipient animals against a second challenge 100 days later. Thus *A. phagocytophilum* appears to persist in small numbers despite the concomitant presence of antibodies and the demonstrable inhibition of leukocyte migration.[109]

CLINICAL FINDINGS

With experimental transmission using fresh blood from an infected horse, the incubation period for equine ehrlichiosis is 1 to 9 days.[91] However, when naturally infected *I. pacificus* ticks are attached, clinical signs appeared at 18 or 25 days after exposure in two horses and one horse remained normal. All three horses were infected as determined by PCR, but sequences of 16S rRNA gene of *A. phagocytophilum* in three horses were different from that of the type strain, indicating strain and disease variations.[102] Clinical signs of disease include fever lasting 1 to 9 days, depression, partial anorexia, limb edema, petechiae, icterus, ataxia, and reluctance to move.[105] In contrast to *N. risticii* infection, laminitis does not develop in equine ehrlichiosis.[110] Experimental inoculation of seven pregnant mares caused clinical signs of various severity, but none of the mares aborted. Hematologic changes observed included thrombocytopenia, decreased packed cell volume, and significant leukopenia, first involving lymphocytes and then granulocytes. The disease is inapparent to mild and is usually not fatal except for injury resulting from ataxia or secondary infection. *A. phagocytophilum* morulae are found in the cytoplasm of neutrophils and eosinophils only during the acute phase of the disease. The infection rate of peripheral blood neutrophils varies from 0.5% to 73%.[91] Chronic cases have not been reported.

DIAGNOSIS

Direct microscopic examination of Romanowsky-stained peripheral blood buffy coat smears (the author prefers Giemsa or Diff-Quik) is simple and inexpensive and provides a permanent record. *A. phagocytophilum* can be seen in granulocytes of buffy coat smears during the acute phase of the disease at 1000 times magnification. When more than three ehrlichial inclusion bodies (morulae) are seen, the diagnosis is considered definitive.[110] Culture isolation of *A. phagocytophilum* from the blood of horses rarely is performed, although *A. phagocytophilum* is isolated routinely from human patients with HGE. An IFA test using the buffy coat cells of the infected horse as the antigen has been developed and has been useful in detecting and titrating antibody in recovered horses.[109] Infected ponies become seropositive by IFA test at 21 days after inoculation, and antibody titers as high as 1:1280 can be detected at day 75 after inoculation. Major surface protein antigens of approximately 44 kd of *A. phagocytophilum* from an HGE patient were cloned in the author's laboratory[111] and others and have been used for serodiagnosis by dot blot Western immunoblot and ELISA of human patients. Because human and horse isolates are highly antigenically cross-reactive, these serologic assays are applicable for diagnosis of equine ehrlichiosis. Nested PCR has been developed for detection of *A. phagocytophilum* 16S rRNA gene in horse blood and ticks.[112] The genes *ank* and *p44* also are used for PCR diagnosis of human HGE patients. None of these methods distinguishes among *A. phagocytophilum* strains (former *E. equi,* the HGE agent, and *E. phagocytophila*) infection.

PATHOLOGIC FINDINGS

The characteristic gross lesions are petechial hemorrhages and edema accompanied by proliferative and necrotizing vasculitis of small arteries and veins in the legs. In mature males, orchitis also may be seen.[91]

THERAPY

Intravenous administration of oxytetracycline at a dosage of 7 mg/kg of body mass is reported to be effective in treating *A. phagocytophilum* infection.[110] After the initial 48 hours of treatment with oxytetracycline, defervescence is seen in all horses treated.

PREVENTION

Because *A. phagocytophilum* most likely is transmitted by *Ixodes* sp. ticks, tick repellent and insecticide are expected to be effective for prevention. A vaccine has not been developed for this disease.

TABLE 2.4-1

Comparison of Biologic Features of *Neorickettsia risticii* and *Anaplasma phagocytophilum*

FEATURE	*N. RISTICII*	*A. PHAGOCYTOPHILUM*
Distribution	United States, Canada, Europe	United States, Europe
Natural host	Horse	Horse, human, sheep, goat, dog, mouse
Experimental host	Mouse, nonhuman primate, cat, dog	Cat, dog, sheep, goat, nonhuman primate but not rat, guinea pig, rabbit, cow
Host cell	Monocyte/macrophage, intestinal epithelial cells, mast cells	Neutrophils, eosinophils
Appearance of inclusion	Individually tightly enveloped by host membrane or densely packed morulae (some of recent Ohio and Kentucky isolates)	Loosely packed small morulae

TABLE 2.4-2

Comparison of Clinical Features of Potomac Horse Fever and Equine Ehrlichiosis

CHARACTERISTIC	POTOMAC HORSE FEVER	EQUINE EHRLICHIOSIS
Mortality	Low to high	None
Acute disease	Yes	Yes
Chronic disease	No	No
Severity	Mild to severe	Mild to moderate
Leukopenia	Yes	Yes
Thrombocytopenia	Yes/no	Yes
Anemia	Yes/no	Yes
Pyrexia; anorexia; depression	Yes	Yes
Laminitis	Yes/no	No
Diarrhea	Yes/no	No
Abortion	Yes/no	No
Ehrlichia	Rarely seen in the blood	Blood granulocytes
Differential diagnosis	Colitis X Salmonellosis Endotoxic shock Antibiotic-associated diarrhea Dietary changes Intoxications	Encephalitis Liver disease Purpura hemorrhagica Equine infectious anemia Equine viral arteritis

Summary

Tables 2.4-1 and 2.4-2 summarize the biologic and clinical features of PHF and equine ehrlichiosis.

In recent years, because of outbreaks of PHF in the United States and discovery of the HGE agent, a strain of *A. phagocytophilum*, awareness has increased of the importance of rickettsial diseases in horses. With rickettsial diseases, wild animals and vector arthropods or helminths are usually reservoirs of rickettsiae, and domestic animals and human beings are accidental dead-end hosts. Because environmental exposure of horses to vectors and reservoirs is high, improving diagnostic, therapeutic, and vaccination procedures is important.

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