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Overview

The Mouse as a Model for Investigation of Human Granulocytic Ehrlichiosis: Current Knowledge and Future Directions

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The use of laboratory mice to investigate correlates of infectious disease, including infection kinetics, cellular alterations, cytokine profiles, and immune response in the context of an intact host has expanded exponentially in the last decade. A marked increase in the availability of transgenic mice and research tools developed specifically for the mouse parallels and enhances this research. Human granulocytic ehrlichiosis (HGE) is an emerging, zoonotic disease caused by tick-borne bacteria. The HGE agent (*Anaplasma phagocytophila*) is one of two recognized pathogens to cause human granulocytic ehrlichiosis (HGE). The mouse model of HGE complements in vitro tissue culture studies, limited in vivo large animal studies, and ex vivo studies of human and ruminant neutrophils, and promises new avenues to approach mechanisms of disease. In the overview reported here, we focus principally on current research into HGE pathogenesis using the mouse model. Included is a discussion of current changes in ehrlichial classification and nomenclature, a review of ehrlichial biology and ecology, and highlights of clinical disease in animals and people.

Human granulocytic ehrlichiosis (HGE) is an emerging, potentially fatal, tick-borne zoonotic disease caused by an obligate intracellular bacterium. These bacteria replicate within the cytoplasm of granulocytes, forming morulae, readily identified by blood smear examination (Fig. 1). Although they were recognized in the 1930s as important animal pathogens (1), granulocytic ehrlichia have only recently been incriminated as human pathogens (2, 3). Indeed, HGE wasn't recognized until 1994 (4). The HGE agent was successfully cultivated in a human promyelocytic leukemia cell line in 1996 (5). As a human syndrome, there has been renewed interest in ehrlichial disease pathogenesis, host immune response, host cell-pathogen interactions, diagnostic assay development, and disease epidemiology.

Animal models of ehrlichial infection have greatly expanded our understanding of ehrlichial biology and disease. This review will focus on the role of the laboratory mouse as a model for HGE, with principal emphasis on disease pathogenesis and host cell-pathogen interactions. Research observations involving use of ex vivo and in vitro cell culture techniques are included to fully delineate advances in understanding HGE pathogenesis. In addition, there is an overview of ehrlichial classification and nomenclature, biology, clinical disease, and ehrlichial infection in other animal species.

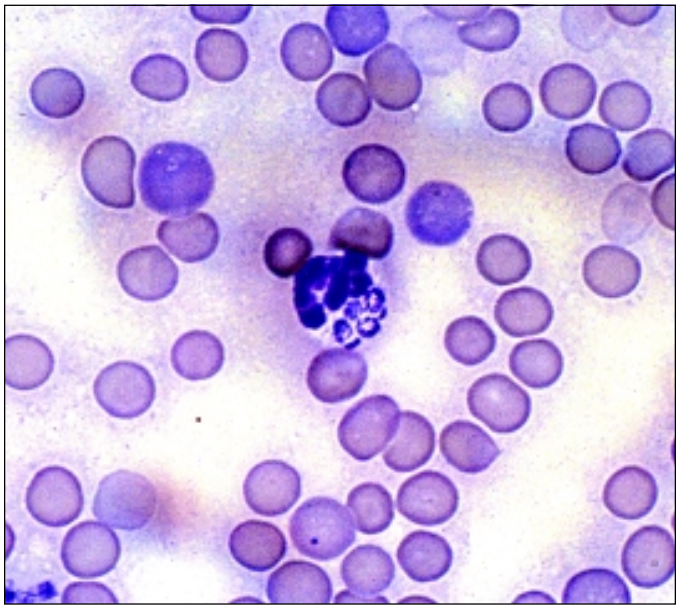

Figure 1. Blood smear from an human granulocytic ehrlichiosis (HGE) agent-infected mouse. Notice that the neutrophil contains pathognomonic large, multiple, intracytoplasmic morulae representing colonies of ehrlichial bacteria.

Classification and Nomenclature

Substantial change in the classification and nomenclature of this group of organisms, on the basis of 16S rRNA gene and *groESL* operon sequences, has taken place. Previously, the *Ehrlichia* genus was in the family Rickettsiaceae and members were classified into three major genogroups on the basis of their 16S rRNA gene sequences (Table 1A). The order Rickettsiales now has two families, Rickettsiaceae and Anaplasmataecae.

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Table 1A. Former classification of ehrlichial organisms

Genogroup	Primary vertebrate host	Vector	Target cell
I.			
E. canis	Canids	*Rhipicephalus sanguineus*	Monocyte, macrophage
E. chaffensis	Humans	*Amblyomma americanum*	Monocyte, macrophage
E. ewingii	Canids, Humans	*Amblyomma americanum*	Neutrophil
E. muris	Mice	*Haemaphusalis sp.*	Monocyte, macrophage
Cowdria ruminantium	Ruminants	*Amblyomma spp.*	Endothelium
II.			
E. phagocytophila	Ruminants	*Ixodes persulcatus* complex	Neutrophil
E. equi	Equids	*Ixodes persulcatus* complex	Neutrophil
HGE	Humans	*Ixodes persulcatus* complex	Neutrophil
LGE*	Llama	*Ixodes pacificus*	Neutrophil
E. platys	Canids	Unknown	Platelet
E. bovis	Ruminants	Unknown	Monocyte
Anaplasma marginale	Ruminants	*Boophilus spp., Dermacentor spp.*	Erythrocyte
III.			
E. sennetsu	Humans	Ingestion of raw fish	Monocyte, macrophage
E. risticii	Equids	Ingestion/snail/fluke	Monocyte, enterocyte
Neorickettsia helmintheca	Canids	Ingestion/salmon/fluke	Macrophage

*LGE = Llama granulocytic *Ehrlichia* sp.

Table 1B. Current classification of the ehrlichial organisms

Genus *Ehrlichia*	Genus *Anaplasma*	Genus *Neorickettsia*
E. canis	*A. phagocytophila**	*N. sennetsu*
E. chaffensis	*A. marginale*	*N. risticii*
E. ewingii	*A. platys*	*N. helminthoeca*
E. muris	*A. bovis*	
E. ruminantium		

*Organisms formerly classified separately as *E. phagocytophila*, *E. equi*, HGE and LGE are now lumped together as one genus and species, *A. phagocytophila*.

Ehrlichia spp., including the HGE agent, are now classified as members of the Anaplasmataceae family (6). All members of this family selectively inhabit and replicate within unique intracellular vacuoles of their host cells, including granulocytes, monocytes, platelets, and erythrocytes. Although these organisms have an ultrastructural similarity to gram-negative bacteria, they lack lipopolysaccarharide endotoxin.

Under the new classification scheme, the former three genogroups have been found to be sufficiently distinct genetically so as to mandate renaming the genogroups as distinct genera (Table 1B). Members of former genogroup I remain within the genus *Ehrlichia*. *Cowdria ruminantium*, which stands alone as an agent that infects endothelium, is now classified as *Ehrlichia ruminantium*. Members of former genogroup II are now within the genus *Anaplasma*. Furthermore, the agents that were formerly named *E. phagocytophila*, *E. equi*, the agent of llama granulocytic ehrlichiosis (LGE), and the HGE agent, which have > 99% homology among them, are now considered to be conspecific, and are named *Anaplasma phagocytophila*. Members of former genogroup III, which share similar fish/fluke life cycles, are now within the genus *Neorickettsia* (Table 1B). Tables 1A and 1B are not complete lists of all members within these families. A comprehensive taxonomic review of the families Rickettsiaceae and Anaplasmataceae has recently been published (6).

The newly created nomenclature provides a cohesive scheme on the basis of clear genetic criteria, and confirms the relatedness of members, yet poses new problems when citing past literature and referring to specific clinical entities. Traditional terminology of these agents, particularly ones within former genogroup II, has been based on the hosts that have been found to harbor them. It is highly unlikely that *E. equi* or the HGE agent, for example, live in nature to infect horses or people, respectively, both of which are likely to be incidental hosts. Tick-borne agents in general have a wide host range. Nevertheless, lumping these agents together among horse and human isolates does not reflect biological differences, such as variable infectivity between horse and human isolates. A somewhat more confusing issue is granulocytic ehrlichiosis, which can be caused not only by the HGE agent, which is now called *A. phagocytophila*, but also by *E. ewingii*, which belongs to the genetically and serologically distinct former genogroup I (2). Thus, these two agents of distinctly different genera induce the same characteristic morulae within granulocytes, infect humans and dogs, and induce an indistinguishable clinical syndrome of granulocytic ehrlichiosis.

The subsequent text of this review deals solely with the HGE agent (now named *A. phagocytophila*). The other known cause of granulocytic ehrlichiosis, *E. ewingii*, has not been successfully cultured, and specific diagnostic assays as well as basic research on this pathogen are notably scant. For the sake of clarity and easy reference to past literature, this review uses traditional terminology to minimize confusion when referring to different cited studies. The HGE agent, therefore, is the term used in this review for human isolates (rather than horse, sheep, or other hosts) that are associated with the human clinical syndrome of HGE. The terms *E. equi* and *E. phagocytophila* will be used to denote experimentally induced or natural infection with agents isolated from horses and ruminants, respectively, acknowledging however that these organisms are not genetically distinct from one another.

Ecology

As denoted in Tables 1A and 1B, agents within the genera *Ehrlichia* and *Anaplasma* are vectored by different ticks. The HGE agent (*A. phagocytophila*) is vectored by ticks of the *Ixodes persulcatus* genospecies complex. Ticks in this genospecies are found circumpolarly throughout the northern hemisphere, with the evolution of a number of different tick species in association with the advancement and recession of the last ice age. During this process, various ecologic niches evolved that supported different reservoir hosts (generally rodents) that were co-infected with a common guild of pathogens that co-evolved with their tick vectors. Each niche evolved with its own *Ixodes* species, in-

cluding *I. persulcatus* in Eurasia, *I. ricinus* in western Europe, *I. scapularis* in the northeastern and upper mid-western United States, *I. pacificus* in the western United States, and many other lesser species. Each niche, regardless of its geographic location, contained a guild of common co-evolving pathogens: *Ehrlichia* (now *Anaplasma*), *Borrelia*, and *Babesia* spp., and tick-borne encephalitis viruses. These agents are co-transmitted among the same reservoir hosts and same ticks within a particular ecosystem. These hosts and ecosystems have merged and mixed since the last ice age due to the environmental impact and traffic of humankind. In Europe, for example, *I. ricinus* ticks that are infected with three distinct genospecies of *B. burgdorferi*, (all agents of Lyme borreliosis), which each originated from either Eurasia, Europe or North America, have been found. Even in North America, single ticks may carry a genetic mix of *B. burgdorferi* isolates. Thus, it is likely that the ehrlichiae in these environments also are likely to be equally diverse. They also are likely to vary in their individual potential for pathogenicity, as has been documented to exist among *B. burgdorferi* genospecies and among various *B. burgdorferi* isolates (7). As human pathogens, the ehrlichiae lag about ten years behind *B. burgdorferi* in scientific awareness, so that we can expect growing clarity on the genetic and biologic diversity of the HGE agent.

Human Disease

HGE is characterized by fever, headache, myalgias, chills, and malaise. Less common symptoms include nausea, anorexia, vomiting, arthralgias, cough, confusion, weakness, diarrhea, pneumonia, vertigo, seizure, and rash (8, 9). Despite the selective granulocytotropism of the HGE agent, hematologic abnormalities include thrombocytopenia, leukopenia (lymphopenia and neutropenia, occasionally with a left shift), and anemia. Later, reactive lymphocytosis and monocytosis are frequently observed (8, 10). Serum biochemical abnormalities may include increased aspartate and alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, and C-reactive protein activities, indicative of mild hepatocellular injury and acute inflammation (8, 9).

Toxic shock-like syndrome (11, 12) that results in pulmonary, renal, cardiac, and neurologic complications, particularly in elderly or debilitated hosts, has been reported (13-16). Consumptive coagulopathy, with increases in prothrombin time, activated partial thromboplastin time, and fibrin(ogen) degradation products, also has been described (17). The diagnostic hallmark of acute HGE is presence of morulae within circulating neutrophils; however, their absence does not rule out infection. Results of polymerase chain reaction (PCR) analysis and paired serologic titers are used to confirm infection.

Granulocytic Ehrlichiosis in Other Species

The first granulocytic ehrlichial organism described was *E. phagocytophila*, the agent of tick-borne fever in ruminants (1). As such, early research focused on the disease in small ruminants, especially sheep. When inoculated intraveneously (i.v.) with infected blood, sheep become febrile and parasitemic by postinoculation days 3 to 4, with bacteremia continuing for 6 to 8 days, as evidenced by circulating morulae (18). Tick-borne fever is characterized by fever, apathy, anorexia, tachypnea, nasal discharge, cough, and reluctance to move (19). Sheep and cattle manifest classic hematologic alterations, including presence of morulae, thrombocytopenia, early leukopenia characterized by lymphopenia and marked neutropenia, as well as monocytosis at postinoculation day 5 (20, 21). Additionally, abortion commonly occurs if naïve pregnant sheep are introduced to pasture infested with vector ticks (*I. ricinus*) (22). The most important clinical consequences of tick-borne fever are secondary infections, including staphylococcal abscesses and septicemia, pasteurellosis, and flavivirus infection (louping ill), among others (23-29). After infection, sheep remain infective for at least 35 days, and possibly as long as 2 years (30). Experimentally induced infection of cows with the HGE agent in Switzerland resulted in seroconversion to the HGE agent and conferred protection from later challenge with *E. phagocytophila*; however, clinical or laboratory signs of acute infection were not observed. In contrast, experimentally induced infection with *E. phagocytophila* resulted in typical clinical and laboratory signs of illness, highlighting the biologic differences between strains and isolates (21).

Granulocytic ehrlichiosis in horses, attributed to *E. equi*, was first described in 1969 (31). Experimentally induced infection of horses via intravenous inoculation results in fever and parasitemia in approximately 5 days. Results of PCR analysis of blood generally are positive by postinoculation day 3 (32). Clinical signs of infection include fever, depression, anorexia, icterus, ataxia, and limb edema (33, 34). Moderate to marked thrombocytopenia, moderate leukopenia, and mild anemia, develop from 6 to 15 days after inoculation. Full recovery is generally evident in 2 to 3 weeks (33, 35). Subclinical infection or infection accompanied by mild clinical signs of disease may be the most frequent outcome of infection in horses (30), as the prevalence of seroreactivity to *E. equi* far exceeds that of detectable disease (36). Experimentally induced infection of horses with *E. phagocytophila*, obtained from cows, resulted in seroconversion and protection against subsequent *E. equi* challenge; however, evidence of active disease was not apparent (21). When inoculated with the HGE agent, horses develop clinical disease that is indistinguishable from disease secondary to *E. equi* infection (32). Histopathologic changes in a group of six horses inoculated with *E. equi* have been reported. Important findings included splenic lymphoid depletion and erythrocyte congestion, normal to hypercellular bone marrow, mild pulmonary perivascular lymphohistiocytic infiltrates and mild periportal mononuclear cell infiltrates, or mild lobular hepatitis with focal apoptotic cells (37).

Canine granulocytic ehrlichiosis was first recognized as a distinct clinical entity in Arkansas in 1971. At the time, it was thought to be a new strain of *E. canis* (the agent of canine monocytic ehrlichiosis) (38). It was not until 1992 that the 16S rRNA gene of this agent was sequenced and found to be unique from that of *E. canis* as well as other members of the *Ehrlichia* genus, and the agent was named *E. ewingii*, in honor of S. A. Ewing (39). Concurrently, it is being established that canine granulocytic ehrlichiosis could also develop secondary to infection with *E. equi* (*A. phagocytophila*). Because of the 20 years between initial recognition and molecular definition of *E. ewingii*, literature describing canine granulocytic ehrlichiosis up until the early 1990s does not distinguish between disease secondary to *E. ewingii* and *E. equi*. Currently, granulocytic ehrlichiosis due to *E. ewingii* in dogs is distinguished from granulocytic

ehrlichiosis due to *E. equi* by finding: characteristic bacterial morulae within granulocytes, higher serum antibody titer to *E. canis* than *E. equi*, and positive results of PCR analysis or DNA sequencing. Disease due to *E. ewingii* has been recognized principally in southern United States, including Missouri, Oklahoma, Tennessee, North Carolina, and Virginia (40-43). This geographic distribution reflects vector distribution and can be used in conjunction with the aforementioned parameters to predict probability of infection with *E. ewingii* versus *E. equi*. Clinically, dogs have evidence of muscular stiffness, lameness or polyarthritis and, rarely, meningitis. They also have the usual hematologic abnormalities, with moderate to severe anemia (41, 42, 44).

As mentioned previously, dogs were found to be experimentally susceptible to *E. equi* in the mid-1970s (45), and in 1982, *E. equi* was found to be associated with naturally infected dogs in California (46). Since then, clinical disease or serologic evidence of infection has been found in numerous locales, including Minnesota, Wisconsin, Connecticut, New York, North Carolina, Virginia, and Sweden (41, 47-49). Disease ranges from mild and subclinical to overt, with fever, depression, lethargy, and classic hematologic alterations. Histopathologic changes include reactive splenic hyperplasia and non-specific mononuclear reactive hepatitis (49).

Laboratory Mice as a Model for HGE

Laboratory mice serve as useful tools for investigation of HGE pathogenesis and infection kinetics, not easily evaluated in people or other large animal species. Soon after recognition of HGE, Telford and co-workers (50) inoculated several mouse strains, including inbred and outbred mice, to examine the deer tick-rodent life cycle of the HGE agent. Most mouse strains were susceptible to infection, as measured by blood smear examination for morulae, PCR analysis, and xenodiagnosis (51). In addition, hematologic, immunologic, and pathologic responses to infection mimicked infection in human beings and other species, although overt clinical signs of disease were not apparent (51, 52). The kinetics of infection have been defined principally in C3H/HeN (C3H) and C57BL/6 (B6) mice. The C3H/HeJ mice have defective macrophage activation and are used only sporadically in studies investigating HGE. As such, C3H mice will be used to refer to studies using C3H/HeN mice, and C3H/HeJ mice will be denoted separately when applicable.

Although B6 mice support a transient and low infection burden, compared with that in C3H mice, they are the background strain for most genetically engineered mice and, thus, are frequently used to model infection kinetics. In contrast to immunocompetent mice that eventually clear infection, C3H mice with severe combined immune deficiency (SCID) remain persistently infected (50). Blood passaged through SCID mice is frequently used as a source of infective material in mouse studies. Thus, infective dose and route of inoculation can be manipulated experimentally, using intraperitoneal injection to standardize dose or exploiting ticks, as mechanical vectors, to study the natural route of infection. Combining the mouse model with quantitative PCR analysis of blood and other tissues permits investigation of infection kinetics not accessible by use of in vitro or cell culture systems. Additionally, use of genetically engineered mice with precise genetic defects, including deletions of leukocyte-signaling molecules, leukocyte adhesion molecules, and cytokines, allows detailed investigation of the correlation between infection kinetics, pathogenesis, and leukocyte function. Table 2 provides a comprehensive list of mouse strains and mutants that have been infected with the HGE agent to investigate disease pathogenesis.

Infected B6 and C3H mice consistently have marked splenomegaly (Fig. 2) on postinfection days 7 to 10. Splenomegaly is secondary to extramedullary hematopoiesis and reactive lymphoid hyperplasia with prominent follicle formation, which subsides by postinfection days 17 to 24. Infected mice also have generalized lymphoid hyperplasia in cervical, mediastinal, and mesenteric lymph nodes as well as Peyer's patches. Active hematopoiesis is evident in the bone marrow of all cell lineages (51). In studies with C3H/HeJ mice, the animals have a few small aggregates of inflammatory cells in the liver as well as rare apoptotic hepatocytes from postinfection days 2 to 14. Extramedullary hematopoiesis is seen in the spleen, with an increase in foamy macrophages. Lymph nodes have paracortical lymphoid hyperplasia, and lung changes consist of mild perivascular infiltrates (53).

Mice infected with the HGE agent can be safely handled after completion of a standard laboratory animal-handling course, with an emphasis on blood-borne pathogens. Direct inoculation, especially needle exposure, is the principal risk of which animal handlers should be made aware. Personal protective equipment, including lab coat, gloves, and booties should be worn. The disease in human beings and animals is readily treated with antibiotics, and the agent is neither air-borne nor transmitted by saliva or fecal/oral routes; thus, research can be performed in a biosafety level-2 laboratory.

Inbred mice can provide consistent, measurable responses to an infection. As such, they have been extensively used in the modeling of infectious disease. However, because of their small size and limited blood volume, mice have been underused in leukocyte function research. Nonetheless, increasingly sensitive analytical techniques now permit single cell analysis. Combined with the availability of genetically modified mouse strains and murine specific monoclonal antibodies in the areas of leukocyte biology and inflammation, these tools provide a rich foundation on which to build in vivo studies of leukocyte function and HGE pathogenesis. The following section focuses on mechanisms by which the HGE agent enters granulocytes, replicates within its own vacuole in a short-lived, terminally differentiated cell, and subverts the powerful bactericidal capacities of their host cells. To date, most host cell-pathogen interactions have been defined in vitro or ex vivo; yet, they set the stage for the pursuit of in vivo pathogenesis in the context of an intact host. Indeed, the mouse model is useful only if it is understood within the context of the overall biology of the agent in a variety of in vivo and in vitro systems.

The HGE Agent: Life Within a Neutrophil

The HGE agent binds specifically to the fucosylated leukocyte P-selectin glycoprotein ligand 1 (PSGL-1) on human neutrophils. Blocking of PSGL-1 with monoclonal antibodies or proteolytic destruction of PSGL-1 prevents adhesion and entry into host cells (54, 55). This receptor-mediated interaction likely is a key determinant in the pathogen's unique tropism for granulocytes. Bacterial binding to PSGL-1 may be the proximate cause of bacterial entry into the host cell, or it may simply be an initial signal leading to downstream events with resultant entry.

Table 2. Laboratory mice used in HGE research

Mouse strain	Description	Infection features	Infection interval	Reference
C3H/HeJ	Defective macrophage activation	Clears infection	10 days* 24 days†	50 51
DBA/2	Widely used inbred strain	Prolonged infection	> 6 weeks	50
CD-1	Outbred Swiss	Clears infection	10 days*	50
CD-1 (splenectomized)		Increased, prolonged bacteremia	> 60 days	50
C3H/HeN	Widely used inbred strain	Clears infection	10 days* 55 days	50 51
C3H/HeN (splenectomized)		Increased, prolonged bacteremia	> 15 days‡	50
C3H/Smn.CIcrHsd/*scid*	Severe combined immune deficiency	Persistent infection	Persistent	50
Peromyscus leucopus	Natural reservoir host	Clears infection	10 days* 14 days†	50 90
BALB/c	Widely used inbred strain	Clears infection	21 days*	76
BALB/c-*Cmkar2*^{tm1Mwm}	Interleukin 8 receptor (CXCR2) K/O	Decreased bacteremia	7 days*	76
C57BL/6	Widely used inbred strain	Clears infection	20 days†	51
B6.129P2-*Tcrb*$^{tm1momTcrd.tm1mom}$	T cell receptor beta/delta K/O, T cell deficient	Increased, prolonged bacteremia	> 30 days‡	94
B6.129S2-*Igh-6*^{tm1Cgn}	Immunoglobulin heavy chain K/O, B cell deficient	Increased, prolonged bacteremia	> 30 days‡	94
B6.129S7-*rag1*^{tm1mom}	Recombination activating gene-1 K/O	Persistent infection	Persistent	94
B6.129Sv-*CD11b*	β_2-integrin, CD11b/CD18 (Mac1) K/O	Moderately increased bacteremia	8 days‡	74
B6.129S7-*Ifng*^{tm1Ts}	IFN gamma K/O	Markedly increased bacteremia	15 days†	73
B6.129P2-*Nos2*^{tm1Lau}	Inducible nitric oxide synthase K/O	Increased bacteremia	12 days†	72
B6.129S6-*Cybb*^{tm1}	gp91 phox K/O	No differences noted	12 days†	72
B6.129S7-*Ifngr*^{tm1}	IFN gamma receptor K/O	Increased bacteremia	12 days†	72
B6.129S7-*IL10*^{tm1Cgn}	Interleukin 10 K/O	No differences noted	15 days†	90

*Reflects minimal infection time, on the basis of absence of morula from blood.
†Reflects minimal infection time, on the basis of negative results of polymerase chain reaction analysis.
‡Not tested beyond this time point.
K/O = knockout.

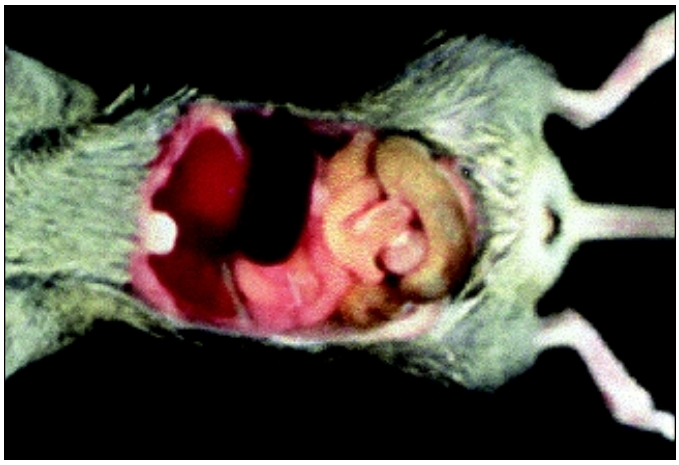

Figure 2. Marked splenomegaly in a C3H/HeN mouse 6 days after intraperitoneal inoculation with the HGE agent.

Either way, it is clear that engagement of PSGL-1 can and likely does lead to signal transduction (56, 57), and that the induced signals may be important not only for entry, but also for intracellular survival. More research is needed to fully define the pathway by which the bacteria enters the cell, the signals transduced along the way that may facilitate survival, the specific bacterial ligands that mediate this interaction, and whether alternate receptors or pathways also are used. To the authors' knowledge, studies evaluating initial bacterial binding and entry into murine neutrophils have not been performed. However, on the basis of known differences in PSGL-1 signal transduction between human and murine neutrophils, research in this area will be vital to understanding comparative HGE pathogenesis (57, 58).

Once inside its host granulocyte, the HGE agent resides within its own unique cytoplasmic membrane-bound vacuole. On the basis of ultrastructural studies of HL-60 cells (a human promyelocytic cell line), vacuoles containing the HGE agent were able to incorporate colloidal gold particles, suggesting these vacuoles were a part of the endocytic pathway (59). However, antibodies directed against the transferrin receptor (an early endosome marker) and lysosomal-associated membrane protein 1 (a late endosome marker), as well as a number of other endosomal markers, did not label HGE agent-containing vacuoles (59, 60). Attempts to label HGE agent-containing vacuoles with mannose-6-phosphate receptor, a marker of endocytic organelles, have yielded discrepant results (59, 60). Co-infection of HL-60 cells with *E. chaffensis* and the HGE agent, and co-incubation of sheep neutrophils with latex beads or *Candida albicans* and the HGE agent, indicated that the HGE agent resides within separate inclusions (61, 62). Thus, it appears that the vacuoles are not typical of either early or late endosomes and, if they are indeed an endosomal compartment, they are somehow modified or fail to mature into phagolysosomes.

As an obligate intracellular pathogen that has chosen to reside within the potentially hostile environment of a powerful antimicrobial cell, simply gaining intracellular access is not sufficient to ensure survival. Usually, pathogens that enter neutrophils via phagocytosis or endocytosis are subject to rapid

destruction once lysosomes fuse with the pathogen-containing phagosome. Prevention of phagosome-lysosome fusion is a mechanism by which a number of pathogens, including *Mycobacterium tuberculosis*, *Legionella pneumophila* and *Neorickettsia risticii*, escape intracellular destruction (63-65). This also appears to be a mechanism by which the HGE agent avoids intracellular destruction. It has been documented in sheep neutrophils and HL-60 cells that vacuoles containing the HGE agent do not fuse with host lysosomes or Golgi-derived vesicles (59, 61, 62). This prevention of phagosome-lysosome fusion requires active synthesis of bacterial proteins, as preincubation of infected sheep neutrophils with oxytetracycline results in phagosome-lysosome fusion (61).

One of the many paradoxes of the HGE agent is that it chooses to occupy a terminally differentiated granulocyte that has a circulating half-life of < 8 h. This life span is clearly too short for ehrlichial replication and formation of the pathognomonic morulae found in circulating neutrophils (Fig. 1). This suggests that the HGE agent either infects granulocytic precursors in bone marrow and the bacteria replicate during granulocyte maturation, and/or that intracellular infection alters the life span of granulocytes. Klein and co-workers (66) observed that isolated human bone marrow stem cells stimulated toward granulocytic differentiation are susceptible to infection with the HGE agent (66). Studies in C3H mice have documented the HGE agent DNA in bone marrow up to 60 days after infection, regardless of the inoculation route (67, 68). Given the high number of susceptible granulocyte precursors in bone marrow, infection in this tissue is not surprising. The tools available for studying hematopoiesis, including lineage specific markers and quantitative colony-forming assays, will permit more detailed investigations into the role primary bone marrow infection plays in the paradox of circulating morulae, pathogenesis of cytopenias and maintenance of long-term infection.

Another possible explanation for the presence of circulating morulae is prolongation of granulocyte survival. Indeed, intracellular infection with the HGE agent inhibited apoptosis of human neutrophils in vitro (69). Delayed apoptosis required binding of the pathogen to a protein component of the host cell membrane and internalization of the HGE agent. However, ehrlichial protein synthesis was not required, as addition of oxytetracycline to the culture did not prevent the anti-apoptotic effect. Although the mechanism behind inhibition of apoptosis was not delineated, the authors found that protein kinase A, nuclear factor-κB, and interleukin 1β were not directly involved (69). Whether delayed apoptosis contributes substantially to bacterial survival and propagation in vivo is not known; however, investigations of neutrophil life span in vivo can be readily evaluated in the mouse model of HGE.

It has long been speculated that the antimicrobial capacities of granulocytes infected with ehrlichiae are altered. In large part this was due to early recognition that *E. phagocytophila* in sheep and cattle exacerbates other diseases, including louping ill, listeriosis, pasteurellosis, chlamydiosis, streptococcal abscesses, and parainfluenza 3 (23-29). Early studies of granulocyte function focused on neutrophils isolated from sheep infected with *E. phagocytophila*. Assays that compared the ex vivo capacity of neutrophils to phagocytose *Staphylococcus aureus* suggested that neutrophils from infected sheep were less able to phagocytose *S. aureus* than were neutrophils from uninfected sheep. In addition, qualitative assays suggested that bacterial killing was also reduced in neutrophils from infected sheep (18).

Neutrophils contain powerful oxygen-dependent and oxygen-independent mechanisms for killing of microbes. The respiratory burst initiated by the NADPH oxidase system is a first line of defense against most invading pathogens. Recently, using diverse models, including human blood leukocytes (neutrophils and monocytes), murine splenic neutrophils, and HL-60 cells, it has been documented that granulocytes infected with the HGE agent do not generate superoxide anion (70, 71). In addition, monocytes, known to be resistant to infection in vivo do not have decreased respiratory burst (71). At least one mechanism behind inhibition of the respiratory burst is the down-regulation of *glycoprotein91phox*, one of four components of the NADPH oxidase system. Using fluorescence-activated cell sorter (FACS) analysis and reverse transcription-PCR (RT-PCR) analysis, decreased expression of *glycoprotein91phox* was observed in infected HL-60 cells and in C3H murine splenic neutrophils in vivo on days 2 and 8 after inoculation. Consistent with these findings, B6, compared with wild-type, mice with a genetic deletion of *glycoprotein91phox* did not experience alterations in infection kinetics or bacterial killing/clearance (72). That report highlights the strength of the mouse model in that it permits the correlation of a phenomenon (decreased superoxide anion production) with alterations in a single gene.

A second important microbiocidal pathway in neutrophils is production of reactive nitrogen intermediates by inducible nitric oxide synthase (iNOS). The role of iNOS in bacterial clearance was investigated by infecting B6-iNOS knockout mice with the HGE agent. These mice had delayed clearance of infection (day 12, compared with day 8), as indicated by results of RT-PCR of splenocytes (72). As interferon (IFN)-γ signaling can induce iNOS, and IFN-γ has been documented to dominate the murine cytokine response to the HGE agent (73), B6 mice with genetic deletion of the IFN-γ receptor also were examined. As with iNOS deficient mice, delayed clearance of the HGE agent was observed (72). The authors concluded that iNOS was likely to be important in early pathogen clearance, and this may be mediated by IFN-γ working via its receptor. However, neither mechanism is necessary for eradication of persistent infection, as both groups of knockout mice were ultimately able to clear infection by day 20. In this instance, use of knockout mice helped to differentiate between the roles of innate (or pre-immune) host defenses and the specific immune response to infection with the HGE agent.

Cellular activation status during infection can be assessed via detection of adhesion molecules on the cell surface. Infection of mice with the HGE agent results in neutrophil activation, as evidenced by up-regulation of the β2 integrin (CD11b/CD18) on the neutrophil surface in vivo (74). This activation occurs in the absence of IFN-γ and is directly associated with intracellular presence of bacteria. Activation occurs in only a subset of circulating neutrophils and, thus, may not be the end result of all bacteria-host cell interactions; however, it may facilitate organism clearance. Clearly, cellular activation is detrimental to pathogen survival, as infection of CD11b/CD18 B6-knockout mice results in an early increase in bacteremia (74). In the end, pathogen survival mandates that multiple facets of the cellular activation cascade be diminished and, indeed, it appears that the HGE agent does just that.

The ability of neutrophils to migrate in tissues toward an in-

flammatory stimulus is mandatory for successful cell function. Due to the HGE agent's unique association with its host cell, neutrophil migration may play many roles in HGE pathogenesis, including cytopenias, bacterial dissemination, and transmission to a competent vector. Studies of the role of chemotaxis and directed neutrophil migration in bacterial dissemination and migration to a tick feeding site are ideally suited to the in vivo mouse model. Using this model, it has been found that the HGE agent is maintained principally in blood, bone marrow, and tissues with high blood flow, especially the spleen (68). In addition, bacterial burden and dissemination can be altered by manipulating leukocyte trafficking (75). Akkoyunlu and co-workers (76) found that the HGE agent was able to induce interleukin 8 (IL-8) secretion and CXCR2 (IL-8 receptor) up-regulation in HL-60 cells treated with retinoic acid (which stimulates differentiation toward granulocytes). They also observed that human neutrophils migrated toward HGE agent-infected cells and that this movement was specifically blocked by antibodies to IL-8. Although mice do not produce IL-8, they do produce granulocyte chemotactic agents that function similarly to IL-8 and utilize the CXCR2 family of receptors. Mice with a targeted genetic disruption of CXCR2 were less susceptible to infection with the HGE agent than were wild-type mice. Those authors hypothesized that the HGE agent exploits the induction of IL-8 (or similar chemotactic agents) and its receptors to facilitate cell-cell pathogen transfer and bacterial dissemination (76).

The mouse model permits examination of leukocyte trafficking to the dermis in an infected host. Given that HGE is a tick-borne disease, the migration of infected neutrophils to the dermis may be enhanced in response to a tick stimulus to facilitate tick acquisition and transmission of the HGE agent to a new host. Use of quantitative PCR of C3H mouse skin has documented that HGE copy numbers rapidly increase at the site of a tick bite within 24 to 48 h after tick attachment, compared with those at non-tick sites (77).

Persistent Infection of the Host

Ehrlichial species able to establish persistent infections in their hosts include *E. canis, A. marginale* and *E. ruminantium* (78-81). In a tick-borne disease, persistence may be a mechanism promoting pathogen acquisition and transmission to a new host. *Anaplasma marginale* is closely related to the HGE agent in that its major surface protein 2 (MSP-2) has a high degree of homology to an MSP of the HGE agent *p44* (82). Polymorphic MSP-2 variants are involved in strain-specific immunity and persistent infection of *Anaplasma* sp. in cattle (83). To the authors' knowledge, emergence of polymorphic variants of HGE agent *p44* has not been described, and the question of persistence with the granulocytotropic ehrlichial organisms has yet to be fully resolved. Two immunosuppressed beagles experimentally infected with the HGE agent had intermittently positive PCR results 5.5 months after inoculation (84). One human patient was found to be PCR test positive approximately 1 month after infection (85). Infection in immunocompetent mice can be prolonged for up to 60 days (51). However, persistent infection with the HGE agent has been clearly documented only in SCID mice that lack functional B and T lymphocytes (50). Use of sensitive molecular tools, including quantitative PCR analysis, in combination with mouse strains that vary in immune competence and immune response to the HGE agent will be useful in approaching the question of persistent infection. Although a natural, clinical syndrome of "chronic granulocytic ehrlichiosis" has never been adequately documented, under experimental conditions of immunosuppression and in mutant mice without functional immune systems, reactivation of a dormant infection or persistent infection may occur.

Disease Pathogenesis

Regardless of host species, granulocytic ehrlichiosis generally results in peripheral pancytopenia. In fact, retrospective studies have found that the likelihood of having acute HGE was inversely related to leukocyte and/or platelet counts. Human patients with leukopenia or thrombocytopenia were 25 times (8) or 5 or 10 times (10) more likely to have HGE than were patients with a normal blood cell count. Nonetheless, mechanisms underling the pathophysiology of erhlichial cytopenias are not well understood. Potential mechanisms include decreased or ineffective hematopoiesis, increased intramedullary destruction (hemophagocytic syndrome), increased peripheral destruction (immune or non-immune mediated mechanisms), decreased cell life span, or altered cellular distribution (endothelial or splenic sequestration).

The most prominent and consistent hallmark of infection is moderate to marked thrombocytopenia, with approximately 50% reduction in circulating platelet numbers 3 to 5 days after infection. Thrombocytopenia is accompanied by an increase in mean platelet volume, supportive of increased peripheral destruction and increased hematopoietic production of platelets. In one study (86), platelet autoantibodies were found in serum samples obtained from human patients during the acute phase of HGE; however, the retrospective study design precluded differentiation between autoantibodies that preceded infection versus those potentially caused by immune stimulation due to HGE. A substantial percentage of patients had autoantibodies on the basis of the criteria set in the study; however, platelet counts were not performed and the timing of autoantibody production in relation to disease was unknown (86).

The C3H and B6 mice infected with the HGE agent have moderate leukopenia, moderate thrombocytopenia, and variable anemia (51, 52). Thrombocytopenia is evident from postinfection days 3 to 7 in B6 and C3H mice (52). The early and rapid (preimmune) decrease in platelet numbers supports increased destruction or sequestration of platelets rather than immune-mediated destruction or decreased hematopoietic production of cells. Further investigation into the kinetics of thrombocytopenia indicated that neither splenic sequestration nor immune-mediated destruction was responsible for the acute decrease in platelet numbers (52).

In cattle with granulocytic ehrlichiosis, lymphopenia is characterized by reduction in circulating B and T lymphocytes ($CD4^+$ and $CD8^+$ cells) (87, 88). Lymphocytes obtained from sheep experimentally infected with *E. phagocytophila*, had reduced reactivity to the mitogens phytohemagglutinin (T cells) and *Escherichia coli* lipopolysaccharide (B cells). The kinetics of reduced lymphocyte reactivity coincided with the period of parasitemia and leukopenia (89). Evaluation of cytokine profiles during infection has led to speculation that alterations in the cytokine mileau may result in cytopenias due to early inflammatory responses and sequestration or destruction of cells (90). Kim and coworkers (91) documented that human blood monocytes produce

the proinflammatory cytokines, IL-1β, tumor necrosis factor α (TNF-α) and IL-6, when incubated with the HGE agent in vitro. They hypothesized that these cytokine alterations may be responsible for the clinical signs of disease and cytopenias associated with HGE. At odds with these findings are those of a study by Klein and co-workers (92) that utilized HL-60 cells stimulated to differentiate into granulocytes and enriched human bone marrow cells as in vitro models for study of cytokine production after HGE agent inoculation. They found induction of CC and CXC chemokines with a notable absence of proinflammatory cytokines IL-1, IL-6, and TNF-α. The chemokines produced were chemoattractants, including, MCP-1, macrophage inflammatory proteins1α and 1β (MIP-1α and -β), and IL-8. These cytokines are also known to be capable of suppressing hematopoiesis (although this was not measured in the study). Those investigators proposed that production of chemokines may suppress hematopoiesis and, thereby, contribute to the cytopenias.

Discrepant results may be due, in part, to the different cell systems used to model HGE agent infection. In addition, although those studies raise interesting points for further investigation, in vitro analysis of cytokine production does not reflect in vivo physiology and cannot be correlated with infection kinetics or cytopenias. Evaluation of cytokine profiles and speculation as to their effect on the pathogenesis of HGE also has been undertaken in the mouse model. Table 3 provides a comprehesive listing of the cytokines that have been measured in all model systems, the source of cytokines assayed, and the alterations noted. Interpreting these findings is difficult as model systems, measurement techniques, and sampling times vary widely. In addition, cytokines are transiently expressed and generally act locally; therefore, measurement in serum or plasma does not necessarily reflect changes in cells and tissues. With these caveats in mind, a few trends are apparent.

The production of systemically acting, pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6, usually produced in high concentration secondary to sepsis or endotoxin, do not appear to be produced in high amounts during HGE infection in vivo; however, they still may play an important, local role during infection (91). Interferon-γ appears to play an important in vivo role in limiting early bacteremia in multiple strains of mice. Specifically, with HGE agent infection in mice, IFN-γ has been proposed to play a role in bacterial clearance, augmentation of killing, and pathologic injury (73, 93). Given the marked bacteremia and minimal pathologic changes seen in IFN-γ knockout mice, some authors have proposed that any pathologic change is related to host inflammatory response rather than the bacterium itself (93). Expression of the chemotactic cytokines IL-8, MIP-1α, and MCP-1 and murine homologues (including KC), is increased, and they may be important in directed neutrophil/HGE agent movement as well as hematopoiesis (76, 92).

Conclusions

The HGE agent is a fascinating intracellular microbe that has evolved to subvert powerful antimicrobial defenses to survive, replicate, and successfully move from infected hosts into its tick vector and back to new hosts. Research into the pathogenesis of HGE has proliferated in the last several years, and use of mouse models has contributed substantially to our knowledge. Modeling permits comparative analysis of natural disease in animals and people with more refined experimental approaches. From such, a consensus of information evolves. Use of inbred and genetically engineered strains of mice allows insight into precise mechanisms of disease and host response in the context of a whole organism. Functional responses secondary to alterations in a single gene can be evaluated. Identification of host and bacterial factors that contribute to differences in clinical expression of disease can be modeled in vivo. Finally, ongoing advances in technology and in genetically modified mice promise additional avenues of research in HGE agent trafficking, host cell-pathogen interactions, mechanisms of cytopenias, and host immune and pre-immune responses to infection. Understanding the steps that permit the HGE agent to be a successful inhabitant of phagocytic cells will ultimately lead to identification of strategies for vaccine development, biological control, and therapeutics to benefit human beings and domestic animals.

References

1. **Gordon, W. S., A. Brownlee, D. R. Wilson, and J. MacLeod.** 1932. Tick-borne fever (a hitherto undescribed disease of sheep). J. Comp. Pathol. **45:**301-312.
2. **Buller, R. S., M. Arens, S. P. Hmiel, C. D. Paddock, J. W. Sumner, Y. Rikhisa, A. Unver, M. Gaudreault-Keener, F. A. Manian, A. M. Liddell, N. Schmulewitz, and G. A. Storch.** 1999. *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. N. Engl. J. Med. **341:**148-155.
3. **Walker, D. H. and J. S. Dumler.** 1996. Emergence of the ehrlichioses as human health problems. Emerg. Infect. Dis. **2:**18-29.
4. **Chen, S. M., J. S. Dumler, J. S. Bakken, and D. H. Walker.** 1994. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J. Clin. Microbiol. **32:**589-595.
5. **Goodman, J. L., C. Nelson, B. Vitale, J. E. Madigan, J. S. Dumler, T. J. Kurtti, and U. G. Munderloh.** 1996. Direct cultivation of the causative agent of human granulocytic ehrlichiosis. N. Engl. J. Med. **334:**209-215.
6. **Dumler, J. S., A. F. Barbet, C. P. Bekker, G. A. Dasch, G. H. Palmer, S. C. Ray, Y. Rikihisa, and F. R. Rurangirwa.** 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int. J. Syst. Evol. Microbiol. **51:**2145-2165.
7. **Barthold, S. W.** 2000. Lyme borreliosis, p. 281-304. *In* J. P. Nataro, M. J. Blaser, and S. Cunningham (ed.), Persistent bacterial infections. ASM Press, Washington, D.C.
8. **Bakken, J. S., J. Krueth, C. Wilson-Nordskog, R. L. Tilden, K. Asanovich, and J. S. Dumler.** 1996. Clinical and laboratory characteristics of human granulocytic ehrlichiosis. Jama **275:**199-205.
9. **Dumler, J. S. and J. S. Bakken.** 1995. Ehrlichial diseases of humans: emerging tick-borne infections. Clin. Infect. Dis. **20:**1102-1110.
10. **Bakken, J. S., M. E. Aguero-Rosenfeld, R. L. Tilden, G. P. Wormser, H. W. Horowitz, J. T. Raffalli, M. Baluch, D. Riddell, J. J. Walls, and J. S. Dumler.** 2001. Serial measurements of hematologic counts during the active phase of human granulocytic ehrlichiosis. Clin. Infect. Dis. **32:**862-870.
11. **Brouqui, P. and D. Raoult.** 1995. Human ehrlichiosis with features of toxic shock syndrome. Am. J. Med. **99:**107.
12. **Fichtenbaum, C. J., L. R. Peterson, and G. J. Weil.** 1993. Ehrlichiosis presenting as a life-threatening illness with features of the toxic shock syndrome. Am. J. Med. **95:**351-357.
13. **Hardalo, C. J., V. Quagliarello, and J. S. Dumler.** 1995. Human granulocytic ehrlichiosis in Connecticut: report of a fatal case. Clin. Infect. Dis. **21:**910-914.

Table 3. Cytokine alterations during HGE infection

Cytokine	Kinetics	Source	Method	Analyte	Ref.
IL-1α	ND on day 6	Infected, HL60 and raHL60 cells	RT-PCR	mRNA	76
	ND at 7 or 24 h	Supernatant of human PMNs infected in vitro	Sandwich ELISA	Protein	76
	ND at 24 or 48 h	HL60 and DMSO/HL60 cells, enriched human BM cells	Sandwich ELISA	Protein	92
	ND on days 0-17	Plasma of infected C3H/HeJ mice	Ag-capture ELISA	Protein	53
	ND on day 6	Infected, HL60 and raHL60 cells	RT-PCR	mRNA	76
IL-1β	ND at 7 or 24 h	Supernatant of human neutrophils infected in vitro	Sandwich ELISA	Protein	76
	Inc. from 2-32 h	Human WBCs infected in vitro	RT-PCR	mRNA	91
	Inc. at 24 h	Supernatant of human WBCs infected in vitro	Capture ELISA	Protein	91
	Inc. at 2 h	Human blood neutrophils infected in vitro	RT-PCR	mRNA	91
IL-2	ND at 2 h	Human WBCs infected in vitro	RT-PCR	mRNA	91
	Small inc. days 5-45	Splenocytes from infected C3H mice	RT-PCR	mRNA	73
	Dec.,compared with uninfected	Supernatant of stimulated C3H mouse splenocytes	Sandwich ELISA	Protein	73
IL-4	Small inc. days 2-45	Splenocytes from infected B6 mice	RT-PCR	mRNA	73
	Mod. inc. days 2-45	Splenocytes from infected B6 (IFN-/-) mice	RT-PCR	mRNA	73
	Dec., compared with uninfected	Supernatant of stimulated B6 mouse splenocytes	Sandwich ELISA	Protein	73
	ND at 24 or 48 h	HL60 and DMSO/HL60 cells, enriched human BM cells	Sandwich ELISA	Protein	92
IL-6	Inc. from 2-16 h	Human WBCs infected in vitro	RT-PCR	mRNA	91
	Inc. at 2 h	Human blood monocytes infected in vitro	RT-PCR	mRNA	91
	Inc. at 24 h	Human WBCs infected in vitro	Capture ELISA	Protein	91
	Small inc. at 24 and 48 h	Infected HL60 cells	Sandwich ELISA	Protein	92
	Mark. inc. at 24 and 48 h	Infected DMSO/HL60 cells	Sandwich ELISA	Protein	92
	Mod. inc. at 24 and 48 h	Enriched human bone marrow cells	Sandwich ELISA	Protein	92
	ND on day 6	Infected HL60 cells	RT-PCR	mRNA	76
IL-8	High at 72 h and day 6	Infected raHL60 cells	RT-PCR	mRNA	76
	Sign. inc. at 24-120 h	Infected raHL60 cells	Sandwich ELISA	Protein	76
	Sign. inc. at 7 and 24 h	Supernatant of human neutrophils infected in vitro	Sandwich ELISA	Protein	76
	Sign. inc. (time unk.)	Infected human sera	Sandwich ELISA	Protein	76
	Weak (25%) induced at 2 h	Human WBCs infected in vitro	RT-PCR	mRNA	91
	No inc. days 0-17	Plasma of infected C3H/HeJ mice	Ag capture ELISA	Protein	53
	Mod. inc. 4 h to 4 days	Supernatant of stimulated B6 mouse splenocytes	Sandwich ELISA	Protein	93
IL-10	Mod. inc. 4 h, day 4 and 21	Supernatant of stimulated B6 (IFN-/-) mouse splenocytes	Sandwich ELISA	Protein	93
	Inc. on days 2-30	Splenocytes from infected C3H, B6 and B6 (IFN-/-) mice	RT-PCR	mRNA	73
	Weak (60%) induced at 2 and 4 h	Human WBCs infected in vitro	RT-PCR	mRNA	91
IL-12	Inc. on days 2-8	Splenocytes from infected C3H mice	RT-PCR	mRNA	73
	Inc. on days 2-15	Splenocytes from infected B6 and B6 (IFN-/-) mice	RT-PCR	mRNA	73
IL-17	ND at 7 or 24 h	Supernatant of human neutrophils infected in vitro	Sandwich ELISA	Protein	76
ENA 78	ND at 7 or 24 h	Supernatant of human neutrophils infected in vitro	Sandwich ELISA	Protein	76
GCP 2	ND at 7 or 24 h	Supernatant of human neutrophils infected in vitro	Sandwich ELISA	Protein	76
GRO-α	ND at 7 or 24 h	Supernatant of human neutrophils infected in vitro	Sandwich ELISA	Protein	76
	Sign. inc. on day 7	Plasma of infected C3H/HeJ mice	Ag-capture ELISA	Protein	53
	Mod. inc. on days 4,7,10	Supernatant of stimumulated B6 mouse splenocytes	Sandwich ELISA	Protein	93
	Mod. inc. on days 4,7,10	Supernatant of stimulated B6 (IL 10-/-)mouse splenocytes	Sandwich ELISA	Protein	93
IFN-γ	Inc. on days 2-30	Splenocytes from infected C3H mice	RT-PCR	mRNA	73
	Sign. inc. on days 2,5	Pooled sera from infected C3H mice	Sandwich ELISA	Protein	73
	Sign. inc. on day 8	Supernatant of stimulated C3H mouse splenocytes	Sandwich ELISA	Protein	73
	Inc. on days 2-45	Splenocytes from infected B6 mice	RT-PCR	mRNA	73
	Sign. inc. on day 8	Supernatant of stimumulated B6 mouse splenocytes	Sandwich ELISA	Protein	73
	Weak (25%), induced at 2 and 4 h	Human WBCs infected in vitro	RT-PCR	mRNA	91
KC	Sign. inc. on day 7	Supernatant of BALB/c mice splenocytes infected ex vivo	Sandwich ELISA	Protein	76
	Small inc. at 24 and 48 h	HL60 cells	Sandwich ELISA	Protein	92
MCP-1	Mark. inc. at 24 and 48 h	DMSO/HL60 cells	Sandwich ELISA	Protein	92
	Mod. inc. at 24 and 48 h	Enriched human BM cells	Sandwich ELISA	Protein	92
	Small inc. at 24 and 48 h	HL60 cells	Sandwich ELISA	Protein	92
MIP1	Mod. inc. at 24 and 48 h	DMSO/HL60 cells	Sandwich ELISA	Protein	92
	Mod. inc. at 24 and 48 h	Enriched human BM cells	Sandwich ELISA	Protein	92
MIP-1	Inc. (data not shown)	HL60 and DMSO/HL60 cells, enriched human BM cells	Sandwich ELISA	Protein	92
MIP-2	Sign. inc. on day 7	Supernatant of BALB/c mice splenocytes infected ex vivo	Sandwich ELISA	Protein	76
NAP-2	ND at 7 or 24 h	Supernatant of human neutrophils infected in vitro	Sandwich ELISA	Protein	76
RANTES	Inc. (data not shown)	HL60 and DMSO/HL60 cells, enriched human BM cells	Sandwich ELISA	Protein	92
TGF-β	Not induced	Human WBCs infected in vitro	RT-PCR	mRNA	91
	ND at 24 or 48 h	HL60 and DMSO/HL60 cells, enriched human BM cells	Sandwich ELISA	Protein	92
	ND on days 0-17	Plasma of infected C3H mice	Ag-capture ELISA	Protein	53
	ND on day 6	Infected, HL60 and raHL60 cells	RT-PCR	mRNA	76
TNF-α	ND at 7 or 24 h	Supernatant of human neutrophils infected in vitro	Sandwich ELISA	Protein	76
	Inc. from 2-8 h	Human WBCs infected in vitro	RT-PCR	mRNA	91
	Inc. at 24 h	Human WBCs infected in vitro	Capture ELISA	Protein	91
	Inc. at 2 h	Human blood monocytes infected in vitro	RT-PCR	mRNA	91

ND = Not detectable; HL60 cells = human promyelocytic leukemia cell line; RT-PCR = reverse transcriptase polymerase chain reaction; Ag = antigen; Inc. = increased; Dec. = decreased; Mod. = moderate; Mark. = marked; Sign. = significant; raHL60 = retinoic acid-treated HL60 cells; C3H = C3H/HeN mice; B6 = C57BL/6 mice; BM= bone marrow; IL = interleukin; ENA-78 = epithelial cell-derived activating peptide 78; GCP = granulocyte colony-stimulating factor; GRO = growth-related oncogene; IFN = interferon; MCP = monocyte chemotactic protein; MIP = macrophage inflammatory protein; NAP= neutrophil activating peptide; TGF = transforming growth factor; and TNF= tumor necrosis factor.

14. **Jahangir, A., C. Kolbert, W. Edwards, P. Mitchell, J. S. Dumler, and D. H. Persing.** 1998. Fatal pancarditis associated with human granulocytic Ehrlichiosis in a 44-year-old man. Clin. Infect. Dis. **27:**1424-1427.
15. **Patel, R. G. and M. A. Byrd.** 1999. Near fatal acute respiratory distress syndrome in a patient with human ehrlichiosis. South Med. J. **92:**333-335.
16. **Pick, N., I. Potasman, C. Strenger, A. Keysary, and I. Schwartz.** 2000. Ehrlichiosis associated vasculitis. J. Intern. Med. **247:**674-678.
17. **Behl, R., M. B. Klein, L. Dandelet, R. R. Bach, J. L. Goodman, and N. S. Key.** 2000. Induction of tissue factor procoagulant activity in myelomonocytic cells inoculated by the agent of human granulocytic ehrlichiosis. Thromb. Haemost. **83:**114-118.
18. **Woldehiwet, Z.** 1987. The effects of tick-borne fever on some functions of polymorphonuclear cells of sheep. J. Comp. Pathol. **97:**481-485.
19. **Pusterla, N. and U. Braun.** 1997. Clinical findings in cows after experimental infection with *Ehrlichia phagocytophila*. Zentralbl. Veterinarmed. A. **44:**385-390.
20. **Woldehiwet, Z.** 1983. Tick-borne fever: a review. Vet. Res. Commun. **6:**163-175.
21. **Pusterla, N., J. B. Pusterla, U. Braun, and H. Lutz.** 1999. Experimental cross-infections with *Ehrlichia phagocytophila* and human granulocytic ehrlichia-like agent in cows and horses. Vet. Rec. **145:**311-314.
22. **Jones, G. L. and I. H. Davies.** 1995. An ovine abortion storm caused by infection with *Cytoecetes phagocytophila*. Vet. Rec. **136:**127.
23. **Foggie, A.** 1951. Studies on the infectious agent of tick-borne fever in sheep. J. Pathol. Bacteriol. **63:**1-15.
24. **Foggie, A.** 1956. The effects of tick-borne fever infection on the susceptiblity of lambs to staphylococci. J. Comp. Pathol. **66:**278-285.
25. **Gronstol, H. and M. J. Ulvand.** 1977. Listeric septicaemia in sheep associated with tick-borne fever (*Ehrlichia phagocytophila*). Acta Vet. Scand. **18:**575-577.
26. **Macleod, J. and W. S. Gordon.** 1933. Studies of tick-borne fever of sheep. Transmission by the tick *Ixodes ricinus* with a description of the disease produced. Parasitology **25:**273-283.
27. **Munro, R., A. R. Hunter, G. MacKenzie, and D. A. McMartin.** 1982. Pulmonary lesions in sheep following experimental infection by *Ehrlichia phagocytophilia* and *Chlamydia psittaci*. J. Comp. Pathol. **92:**117-129.
28. **Gilmour, J. L., T. A. Brodie, and P. H. Holmes.** 1982. Tick-borne fever and pasteurellosis in sheep. Vet. Rec. **111:**512.
29. **Batungbacal, M. R., and G. R. Scott.** 1982. Tick-borne fever and concurrent parainfluenza-3 virus infection in sheep. J. Comp. Pathol. **92:**415-428.
30. **Ogden, N. H., Z. Woldehiwet, and C. A. Hart.** 1998. Granulocytic ehrlichiosis: an emerging or rediscovered tick-borne disease? J. Med. Microbiol. **47:**475-82.
31. **Gribble, D. H.** 1969. Equine ehrlichiosis. J. Am. Vet. Med. Assoc. **155:**462-469.
32. **Madigan, J. E., P. J. Richter, Jr., R. B. Kimsey, J. E. Barlough, J. S. Bakken, and J. S. Dumler.** 1995. Transmission and passage in horses of the agent of human granulocytic ehrlichiosis. J. Infect. Dis. **172:**1141-1144.
33. **Madigan, J. E., and D. Gribble.** 1987. Equine ehrlichiosis in northern California: 49 cases (1968-1981). J. Am. Vet. Med. Assoc. **190:**445-458.
34. **Madigan, J. E., J. E. Barlough, J. S. Dumler, N. S. Schankman, and E. DeRock.** 1996. Equine granulocytic ehrlichiosis in Connecticut caused by an agent resembling the human granulocytotropic ehrlichia. J. Clin. Microbiol. **34:**434-435.
35. **Stannard, A. A., D. H. Gribble, and R. S. Smith.** 1969. Equine ehrlichiosis: a disease with similarities to tick-borne fever and bovine petechial fever. Vet. Rec. **84:**149-150.
36. **Madigan, J. E., S. Hietala, S. Chalmers, and E. DeRock.** 1990. Seroepidemiologic survey of antibodies to *Ehrlichia equi* in horses of northern California. J. Am. Vet. Med. Assoc. **196:**1962-1964.
37. **Lepidi, H., J. E. Bunnell, M. E. Martin, J. E. Madigan, S. Stuen, and J. S. Dumler.** 2000. Comparative pathology, and immunohistology associated with clinical illness after Ehrlichia phagocytophila-group infections. Am. J. Trop. Med. Hyg. **62:**29-37.
38. **Ewing S. A., R. G. Buckner, and C. S. Hayat.** 1971. A new strain of *Ehrlichia canis*. J. Am. Vet. Med. Assoc. **159:**1771-1774.
39. **Anderson, B. E., C. E. Greene, D. C. Jones, and J. E. Dawson.** 1992. *Ehrlichia ewingii* sp. nov., the etiologic agent of canine granulocytic ehrlichiosis. Int. J. Syst. Bacteriol. **42:**299-302.
40. **Bellah, J. R., R. M. Shull, and E. V. Selcer.** 1986. *Ehrlichia canis*-related polyarthritis in a dog. J. Am. Vet. Med. Assoc. **189:**922-923.
41. **Goldman, E. E., E. B. Breitschwerdt, C. B. Grindem, B. C. Hegarty, J. J. Walls, and J. S. Dumler.** 1998. Granulocytic ehrlichiosis in dogs from North Carolina and Virginia. J. Vet. Intern. Med. **12:**61-70.
42. **Stockham, S. L., D. A. Schmidt, K. S. Curtis, B. G. Schauf, J. W. Tyler, and S. T. Simpson.** 1992. Evaluation of granulocytic ehrlichiosis in dogs of Missouri, including serologic status to *Ehrlichia canis*, *Ehrlichia equi*, and *Borrelia burgdorferi*. Am. J. Vet. Res. **53:**63-68.
43. **Cowell, R. L., R. D. Tyler, K. D. Clinkenbeard, and J. H. Meinkoth.** 1988. Ehrlichiosis and polyarthritis in three dogs. J. Am. Vet. Med. Assoc. **192:**1093-1095.
44. **Maretzki, C. H., D. J. Fisher, and C. E. Greene.** 1994. Granulocytic ehrlichiosis and meningitis in a dog. J. Am. Vet. Med. Assoc. **205:**1554-1556.
45. **Lewis, G. E., Jr., D. L. Huxsoll, M. Ristic, and A. J. Johnson.** 1975. Experimentally induced infection of dogs, cats, and nonhuman primates with *Ehrlichia equi*, etiologic agent of equine ehrlichiosis. Am. J. Vet. Res. **36:**85-88.
46. **Madewell, B. R. and D. H. Gribble.** 1982. Infection in two dogs with an agent resembling *Ehrlichia equi*. J. Am. Vet. Med. Assoc. **180:**512-514.
47. **Greig, B., K. M. Asanovich, P. J. Armstrong, and J. S. Dumler.** 1996. Geographic, clinical, serologic, and molecular evidence of granulocytic ehrlichiosis, a likely zoonotic disease, in Minnesota and Wisconsin dogs. J. Clin. Microbiol. **34:**44-48.
48. **Magnarelli, L. A., J. W. Ijdo, J. F. Anderson, J. E. Madigan, J. S. Dumler, and E. Fikrig.** 1997. Antibodies to *Ehrlichia equi* in dogs from the northeastern United States. J Am. Vet. Med. Assoc. **211:**1134-1137.
49. **Egenvall, A., A. Bjoersdorff, I. Lilliehook, E. Olsson Engvall, E. Karlstam, K. Artursson, A. Hedhammar, and A. Gunnarsson.** 1998. Early manifestations of granulocytic ehrlichiosis in dogs inoculated experimentally with a Swedish *Ehrlichia* species isolate. Vet. Rec. **143:**412-417.
50. **Telford, S. R., 3rd, J. E. Dawson, P. Katavolos, C. K. Warner, C. P. Kolbert, and D. H. Persing.** 1996. Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. Proc. Natl. Acad. Sci. USA **93:**6209-6214.
51. **Hodzic, E., J. W. Ijdo, S. Feng, P. Katavolos, W. Sun, C. H. Maretzki, D. Fish, E. Fikrig, S. R. Telford, 3rd, and S. W. Barthold.** 1998. Granulocytic ehrlichiosis in the laboratory mouse. J. Infect. Dis. **177:**737-745.
52. **Borjesson, D. L., S. I. Simon, F. Tablin, and S. W. Barthold.** 2001. Thrombocytopenia in a mouse model of human granulocytic ehrlichiosis. J. Infect. Dis. **184:**1475-1479.
53. **Martin, M. E., J. E. Bunnell, and J. S. Dumler.** 2000. Pathology, immunohistology, and cytokine responses in early phases of human granulocytic ehrlichiosis in a murine model. J. Infect. Dis. **181:**374-378.
54. **Herron, M. J., C. M. Nelson, J. Larson, K. R. Snapp, G. S. Kansas, and J. L. Goodman.** 2000. Intracellular parasitism by the human granulocytic ehrlichiosis bacterium through the P-selectin ligand, PSGL-1. Science **288:**1653-1656.
55. **Goodman, J. L., C. M. Nelson, M. B. Klein, S. F. Hayes, and B. W. Weston.** 1999. Leukocyte infection by the granulocytic ehrlichiosis agent is linked to expression of a selectin ligand. J. Clin. Invest. **103:**407-412.
56. **McEver, R. P. and R. D. Cummings.** 1997. Perspectives series: cell adhesion in vascular biology. Role of PSGL-1 binding to selectins in leukocyte recruitment. J. Clin. Invest. **100:**485-491.

57. **Yang, J., B. C. Furie, and B. Furie.** 1999. The biology of P-selectin glycoprotein ligand-1: its role as a selectin counterreceptor in leukocyte-endothelial and leukocyte-platelet interaction. Thromb. Haemost. **81:**1-7.
58. **Blanks, J. E., T. Moll, R. Eytner, and D. Vestweber.** 1998. Stimulation of P-selectin glycoprotein ligand-1 on mouse neutrophils activates beta 2-integrin mediated cell attachment to ICAM-1. Eur. J. Immunol. **28:**433-443.
59. **Webster, P., L. M. Chicoine, and E. Fikrig.** 1998. The agent of human granulocytic ehrlichiosis resides in an endosomal compartment. J. Clin. Invest. **101:**1932-1941.
60. **Mott, J., R. E. Barnewall, and Y. Rikihisa.** 1999. Human granulocytic ehrlichiosis agent and *Ehrlichia chaffeensis* reside in different cytoplasmic compartments in HL-60 cells. Infect. Immun. **67:**1368-1378.
61. **Gokce, H. I., G. Ross, and Z. Woldehiwet.** 1999. Inhibition of phagosome-lysosome fusion in ovine polymorphonuclear leucocytes by *Ehrlichia (Cytoecetes) phagocytophila*. J. Comp. Pathol.**120:**369-381.
62. **Mott, J., R. E. Barnewall, and Y. Rikihisa.** 1999. Human granulocytic ehrlichiosis agent and *Ehrlichia chaffeensis* reside in different cytoplasmic compartments in HL-60 cells. Infect. Immun. **67:**1368-1378.
63. **Bozue, J. A. and W. Johnson.** 1996. Interaction of *Legionella pneumophila* with *Acanthamoeba castellanii*: uptake by coiling phagocytosis and inhibition of phagosome-lysosome fusion. Infect Immun. **64:**668-773.
64. **Clemens, D. L. and M. A. Horwitz.** 1996. The *Mycobacterium tuberculosis* phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. J. Exp. Med. **184:**1349-1355.
65. **Wells, M. Y. and Y. Rikihisa.** 1988. Lack of lysosomal fusion with phagosomes containing *Ehrlichia risticii* in P388D1 cells: abrogation of inhibition with oxytetracycline. Infect Immun. **56:**3209-3215.
66. **Klein, M. B., J. S. Miller, C. M. Nelson, and J. L. Goodman.** 1997. Primary bone marrow progenitors of both granulocytic and monocytic lineages are susceptible to infection with the agent of human granulocytic ehrlichiosis. J. Infect Dis. **176:**1405-1409.
67. **Bunnell, J. E., L. A. Magnarelli, and J. S. Dumler.** 1999. Infection of laboratory mice with the human granulocytic ehrlichiosis agent does not induce antibodies to diagnostically significant *Borrelia burgdorferi* antigens. J. Clin. Microbiol. **37:**2077-2079.
68. **Hodzic, E., S. Feng, D. Fish, C. M. Leutenegger, K. J. Freet, and S. W. Barthold.** 2001. Infection of mice with the agent of human granulocytic ehrlichiosis after different routes of inoculation. J. Infect. Dis. **183:**1781-1786.
69. **Yoshiie, K., H. Y. Kim, J. Mott, and Y. Rikihisa.** 2000. Intracellular infection by the human granulocytic ehrlichiosis agent inhibits human neutrophil apoptosis. Infect. Immun. **68:**1125-1133.
70. **Banerjee, R., J. Anguita, D. Roos, and E. Fikrig.** 2000. Cutting edge: infection by the agent of human granulocytic ehrlichiosis prevents the respiratory burst by down-regulating gp91phox. J. Immunol. **164:**3946-3949.
71. **Mott, J. and Y. Rikihisa.** 2000. Human granulocytic ehrlichiosis agent inhibits superoxide anion generation by human neutrophils. Infect. Immun. **68:**6697-6703.
72. **Banerjee, R., J. Anguita, and E. Fikrig.** 2000. Granulocytic ehrlichiosis in mice deficient in phagocyte oxidase or inducible nitric oxide synthase. Infect. Immun. **68:**4361-4362.
73. **Akkoyunlu, M. and E. Fikrig.** 2000. Gamma interferon dominates the murine cytokine response to the agent of human granulocytic ehrlichiosis and helps to control the degree of early rickettsemia. Infect. Immun. **68:**1827-1833.
74. **Borjesson, D. L., S. I. Simon, E. Hodzic, C. M. Ballantyne, and S. W. Barthold.** 2002. Kinetics of CD11b/CD18 upregulation during infection with the agent of human granulocytic ehrlichiosis in mice. Lab. Invest. **82:**303-311.
75. **Borjesson, D. L., S. I. Simon, E. Hodzic, H. E. V. DeCock, C. M. Ballantyne, and S. W. Barthold.** 2002. Roles of neutrophil B2 integrins in kinetics of bacteremia, extravasation and tick acquisition of *Anaplasma phagocytophila* in mice. Blood (In review).
76. **Akkoyunlu, M., S. E. Malawista, J. Anguita, and E. Fikrig.** 2001. Exploitation of interleukin-8-induced neutrophil chemotaxis by the agent of human granulocytic ehrlichiosis. Infect. Immun. **69:**5577-5588.
77. **Hodzic, E., D. L. Borjesson, S. Feng and S.W. Barthold.** 2001. Acquisition dynamics of *Borrelia burgdorferi* and the agent of human granulocytic ehrlichiosis at the host- vector interface. Vector Borne Zoonot. Dis. **1:**149-158.
78. **Iqbal, Z. and Y. Rikihisa.** 1994. Reisolation of *Ehrlichia canis* from blood and tissues of dogs after doxycycline treatment. J. Clin. Microbiol. **32:**1644-1649.
79. **Barbet, A. F., S. M. Semu, N. Chigagure, P. J. Kelly, F. Jongejan, and S. M. Mahan.** 1994. Size variation of the major immunodominant protein of *Cowdria ruminantium*. Clin. Diagn. Lab. Immunol. **1:**744-746.
80. **Brouqui, P., J. S. Dumler, D. Raoult, and D. H. Walker.** 1992. Antigenic characterization of ehrlichiae: protein immunoblotting of *Ehrlichia canis*, *Ehrlichia sennetsu*, and *Ehrlichia risticii*. J. Clin. Microbiol. **30:**1062-1066.
81. **Eriks, I. S., G. H. Palmer, T. C. McGuire, D. R. Allred, and A. F. Barbet.** 1989. Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. J. Clin. Microbiol **27:**279-284.
82. **Ijdo, J. W., W. Sun, Y. Zhang, L. A. Magnarelli, and E. Fikrig.** 1998. Cloning of the gene encoding the 44-kilodalton antigen of the agent of human granulocytic ehrlichiosis and characterization of the humoral response. Infect. Immun. **66:**3264-3269.
83. **French, D. M., W. C. Brown, and G. H. Palmer.** 1999. Emergence of *Anaplasma marginale* antigenic variants during persistent rickettsemia. Infect Immun. **67:**5834-5840.
84. **Egenvall, A., I. Lilliehook, A. Bjoersdorff, E. O. Engvall, E. Karlstam, K. Artursson, M. Heldtander, and A. Gunnarsson.** 2000. Detection of granulocytic *Ehrlichia* species DNA by PCR in persistently infected dogs. Vet. Rec. **146:**186-190.
85. **Dumler, J. S. and J. S. Bakken.** 1996. Human granulocytic ehrlichiosis in Wisconsin and Minnesota: a frequent infection with the potential for persistence. J. Infect Dis. **173:**1027-1030.
86. **Wong, S. J. and J. A. Thomas.** 1998. Cytoplasmic, nuclear, and platelet autoantibodies in human granulocytic ehrlichiosis patients. J. Clin Microbiol. **36:**1959-1963.
87. **Batungbacal, M. R., G. R. Scott, and C. Burrells.** 1982. The lymphocytopaenia in tick-borne fever. J. Comp. Pathol. **92:**403-407.
88. **Woldehiwet, Z.** 1991. Lymphocyte subpopulations in peripheral blood of sheep experimentally infected with tick-borne fever. Res. Vet Sci. **51:**40-43.
89. **Woldehiwet, Z.** 1987. Depression of lymphocyte response to mitogens in sheep infected with tick-borne fever. J. Comp. Pathol. **97:**637-643.
90. **Bunnell, J. E., E. R. Trigiani, S. R. Srinivas, and J. S. Dumler.** 1999. Development and distribution of pathologic lesions are related to immune status and tissue deposition of human granulocytic ehrlichiosis agent-infected cells in a murine model system. J. Infect. Dis. **180:**546-550.
91. **Kim, H. Y. and Y. Rikihisa.** 2000. Expression of interleukin-1beta, tumor necrosis factor alpha, and interleukin-6 in human peripheral blood leukocytes exposed to human granulocytic ehrlichiosis agent or recombinant major surface protein P44. Infect. Immun. **68:**3394-3402.
92. **Klein, M. B., S. Hu, C. C. Chao, and J. L. Goodman.** 2000. The agent of human granulocytic ehrlichiosis induces the production of myelosuppressing chemokines without induction of proinflammatory cytokines. J. Infect. Dis **182:**200-205.
93. **Martin, M. E., K. Caspersen, and J. S. Dumler.** 2001. Immunopathology and ehrlichial propagation are regulated by interferon-gamma and interleukin-10 in a murine model of human granulocytic ehrlichiosis. Am. J. Pathol. **158:**1881-1888.
94. **Hodzic, E., S. Feng, and S. W. Barthold.** Unpublished data.