

COMPARATIVE PATHOLOGY AND IMMUNOHISTOLOGY ASSOCIATED WITH CLINICAL ILLNESS AFTER *EHRlichia PHAGOCYTOPHILA*-GROUP INFECTIONS

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Abstract. The *Ehrlichia phagocytophila*-group also includes *E. equi* and the human granulocytic ehrlichiosis (HGE) agent that are probably a single species. Disease is mild to severe illness in ruminants, horses, and humans, but the comparative pathology and ehrlichial distribution in tissues is poorly described. We compared pathology and ehrlichial distribution in humans with HGE, horses with *E. equi* infection, and a sheep with *E. phagocytophila* infection. Frequent findings included splenic lymphoid depletion, small macrophage aggregates and apoptoses in liver, and paracortical hyperplasia in lymph nodes. Bone marrow was normocellular or hypercellular. Only the spleen was frequently infected; other organs with infected cells included lung, liver, heart, and kidney, but lesions were present in lung and liver only. Most infected cells were neutrophils. *Ehrlichia phagocytophila*-group infections are associated with moderate tissue damage. While the pathogenesis of granulocytic ehrlichiosis is not clear, pathologic studies suggest that the process is initiated by ehrlichia-infected cells but may result from host-mediated injury and immunosuppression.

Ehrlichioses are rickettsial infections that occur in animals and humans and are caused by microorganisms of the genus *Ehrlichia*. The genus *Ehrlichia* belongs to the family *Rickettsiaceae* and consists of small obligatory intracellular bacteria that average 0.5–1.5 μm in length, which proliferate in vacuoles in the cytoplasm of leukocytes and other cells of bone marrow or mesodermal origin.¹ The *Ehrlichia phagocytophila*-group includes *E. phagocytophila*, *E. equi*, and the human granulocytic ehrlichia. The prototype for the *E. phagocytophila* genogroup (a cluster of closely related bacteria based upon gene sequences) is *E. phagocytophila*, a European pathogen of ruminant neutrophils that causes tick-borne fever in sheep, goats, and cattle.² *Ehrlichia equi* is an agent of both equine and canine granulocytic ehrlichiosis.^{3,4} Homology of the 16S ribosomal DNA of these organisms, as well as that of the newly identified agent of human granulocytic ehrlichiosis (HGE), is more than 99.8%.^{5–8} These species are phylogenetically and antigenically very similar.^{5,9} In addition, the HGE agent produces a disease in the horse similar from that caused by *E. equi*¹⁰ and infection of horses with the agent of HGE confers resistance to *E. equi* challenge.¹¹

The causative agent of tick-borne fever was first discovered in Scotland in 1932;¹² however, the disease has probably been recorded in sheep for more than 200 years in Norway.¹³ The disease is characterized by fever, leukopenia, marked neutropenia, and thrombocytopenia.¹⁴ Equine granulocytic ehrlichiosis has been a recognized disease of horses in California since the 1960s.¹⁵ Clinical manifestations include fever, lethargy, anorexia, distal limb edema, petechiation, icterus, ataxia, reluctance to move, and thrombocytopenia.¹⁶ Human granulocytic ehrlichiosis is a recently described disease characterized by fever, headache, myalgia, chills, various combinations of leukopenia, anemia, and thrombocyto-

penia, and mild to significant elevations in levels of aspartate (AST) and alanine aminotransferases (ALT).^{5,17} Ticks are the vectors and small mammals and deer are the likely reservoirs.^{18–21} Severe cases of HGE in humans, when left untreated, may be fatal.^{22–24}

The major infected target cells during the *E. phagocytophila*-group infections are granulocytic leukocytes, mostly neutrophils.²⁴ Diagnostic laboratory testing includes microscopic examination of Wright-stained peripheral blood smears for presence of neutrophilic morulae, polymerase chain reaction (PCR) analysis of blood samples for the *E. phagocytophila*-group DNA, and evaluation of serologic responses by indirect immunofluorescent antibody assay using *E. equi* or *in vitro*-cultivated HGE agent as antigen.^{5,17,24,25} Despite advances in diagnostic tests and increasing molecular characterization of the *E. phagocytophila*-group, little is known about the pathogenesis of infection caused by these ehrlichiae. Because of the similar clinical presentations and evidence of potential conspecificity of the causative agents, we compared the pathology and localization of the ehrlichiae in their respective human-, equine-, and ruminant-infected hosts.

MATERIALS AND METHODS

Patients and tissue specimens. Human tissues were obtained from 4 autopsies, 2 bone marrow biopsies, and 2 lymph node biopsies (1 patient) of hospitalized patients.^{5,22–24,26} All patients had HGE confirmed by demonstration of typical cytoplasmic ehrlichial morulae in neutrophils on blood smears, PCR, and/or serology. *Ehrlichia chaffeensis* infection was excluded in all patients by negative results with PCR assays, negative or 4-fold lower antibody titers, or specific immunohistochemical stains. Four of the 7 pa-

tients from whom tissues for examination were obtained acquired HGE in Wisconsin or Minnesota; the remaining 3 patients acquired the infection in the northeastern United States. Formalin-fixed and paraffin-embedded tissues that were obtained as part of the autopsy or diagnostic evaluation were used for further histologic and immunohistologic examination. All studies of human patients were conducted in accordance with approval by the Joint Council on Clinical Investigation of the Johns Hopkins University School of Medicine.

The experimental horse infections were performed as part of an earlier investigation when *E. equi* was first identified¹⁵ and formalin-fixed, paraffin-embedded tissues were retrieved from the University of California, Davis Veterinary Pathology archives. Briefly, infection was established by intravenous inoculation of blood stabilates from an *E. equi*-infected horse from northern California and resulted in typical equine granulocytic ehrlichiosis, as previously described.^{3,15} Tissues obtained from 6 horses that were killed and necropsied during the acute phase of the infection, ranging from post-inoculation days 10–18 (days 5–11 of fever).

To examine the histopathologic changes during the acute phase of ovine tick-borne fever, a 9-month-old lamb was experimentally infected by intravenous inoculation with 1 ml of a whole blood dimethylsulfoxide stabilate of *E. phagocytophila* originally isolated from a local sheep flock in Norway. The animal was killed and necropsied on day 5 post-inoculation, day 2 of fever. Formalin-fixed, paraffin-embedded tissues were prepared for histologic and immunohistologic examination.

Histologic examination was performed on spleen, liver, lung, lymph node, bone marrow, brain, heart, kidney, and skeletal muscle and tendon, when available. Tissues were stained with hematoxylin and eosin.

All animal experimental infections were performed in compliance with local or NIH guidelines regarding the humane maintenance and care of experimental animals in medical research, and where appropriate, were approved by the Institutional Animal Care and Use Committee.

Immunohistologic detection of *Ehrlichia*. Immunohistology was performed using a protocol derived from that of Cartun and Pederson.²⁷ Briefly, 4 μm -thick tissue sections were prepared on silane-coated slides (Sigma, St. Louis, MO) and heated to 45°C for 30 min prior to deparaffinization in xylene. The slides were then rehydrated in graded alcohol for 2 min each and rinsed in phosphate-buffered saline (PBS), pH 7.4, for 5 min. Tissues were then blocked for 10 min at room temperature with 5% normal goat serum in PBS with 0.5% non-fat dry milk (PBSM). Excess blocking solution was drained and replaced with either polyclonal rabbit anti-HGE agent serum for horse and sheep tissues or horse anti-*E. equi* serum for human tissues. The polyclonal rabbit serum was diluted at 1:200 whereas the polyclonal horse serum was diluted at 1:50, both in PBSM with 5% normal goat serum, and incubated on tissue sections for 1 hr at room temperature. Slides were washed by two brief rinses in PBS and one 5-min immersion in PBS. Subsequently, slides were incubated for 30 min at room temperature with, respectively, biotinylated goat anti-rabbit or anti-horse IgG (Kierkegaard and Perry Laboratories, Gaithersburg, MD) diluted at 1:100 in PBSM with 5% normal goat serum. After washing, the

slides were then incubated for 30 min at room temperature in a 1:50 dilution of alkaline phosphatase-conjugated streptavidin (Dako, Santa Barbara, CA). The slides were again washed in PBS and incubated for 10 min in fast red/naphthol phosphate chromogen solution (Sigma) for alkaline phosphatase conjugates. The unreacted chromogen was removed by immersion in running tap water, and the slides were counterstained with Mayer's hematoxylin for 10 min, blued in Scott's solution for 10 sec, and mounted with Crystal Mount (Biomedex, Foster City, CA). When the slides were dry, they were mounted with glass coverslips and Permount (Fisher, Chicago, IL). For each case, identical tissue sections were prepared for negative controls by substituting immune serum by normal rabbit or horse serum, respectively.

RESULTS

The human patients had clinical evidence of severe HGE with fever, significant headache, myalgia, malaise, and other systemic findings; leukopenia, thrombocytopenia, and elevated AST or ALT were present in most.

Patient 1. An 80-year-old man from Wisconsin developed multiorgan failure, shock, and gastrointestinal hemorrhage and died within 3 weeks of tick bite and 2 weeks of illness in the absence of anti-ehrlichial therapy.²² Three days before his death, a blood smear examination revealed that 41% of peripheral blood neutrophils contained ehrlichial morulae; retrospective examination of blood obtained at this time was PCR-positive.

Patient 2. An 80-year-old man from Minnesota had a previous diagnosis of chronic lymphocytic leukemia and was initially admitted for Richter's syndrome with evidence of supervening large cell lymphoma and was treated with prednisone;^{5,24} diagnosis was based upon ehrlichial morulae present in 16% of peripheral blood neutrophils and PCR, and he was treated with doxycycline for several days prior to death.

Patient 3. A 71-year-old man from Connecticut had radiation therapy for prostate carcinoma 6 months before he presented with clinical manifestations of HGE and morulae in neutrophils on a peripheral blood examination; with intensive support, the patient survived for approximately 1 month.²³ The cause of death was invasive pulmonary aspergillosis, but sera obtained during his acute illness and prior to death revealed a seroconversion for antibodies to *E. equi*.

Patient 4. A 44-year-old man from Minnesota with no significant prior medical history developed clinical findings of HGE within 2 weeks of a tick bite. He was empirically treated for Lyme disease with amoxicillin, but died suddenly during the acute phase of illness. The diagnosis of HGE was established by the demonstration of a ≥ 256 titer of antibodies to *E. equi* and the presence of HGE agent nucleic acids by PCR in the blood at the time of death.²⁶ Serologic test results for *Borrelia burgdorferi* and *Babesia microti* were negative. The cause of death was myocarditis of uncertain etiology.

Patient 5. A 9-year-old boy from upstate New York presented with fever, lymphadenopathy, rash, and thrombocytopenia. To exclude a hematologic malignancy, 2 lymph nodes biopsies were obtained separated by an interval of 20 days. The patient died 32 days after the initial lymph node biopsy. Diagnosis was suspected when a single *E. equi* titer

of 320 was obtained prior to the patient's death. A post-mortem examination was not performed.

Patient 6. An 80-year-old man from Minnesota presented with 21 days of fever, headache, myalgia, nausea, and vomiting, and was discovered to have leukopenia, anemia, and thrombocytopenia. A bone marrow examination was performed to exclude a hematologic malignancy or occult infection. Human granulocytic ehrlichiosis was diagnosed when 3% of peripheral blood neutrophils were found to contain ehrlichial morulae; *E. equi* nucleic acids were identified in blood by PCR, and serum contained antibodies to *E. equi* at a titer of 320. Treatment with doxycycline led to defervescence and recovery.

Patient 7. A 34-year-old woman from Connecticut presented with severe abdominal pain after several days of headache, malaise, myalgia, and intermittent low-grade fever about 2 weeks after removing an attached tick. An emergent appendectomy was performed; however, thereafter she was noted to have progressively worsening thrombocytopenia, leukopenia, anemia, and elevations in AST and ALT. A bone marrow biopsy was obtained to investigate the worsening pancytopenia. Intracytoplasmic inclusions suggestive of ehrlichial morulae were identified and the early convalescent serum had an antibody titer to *E. equi* of 80. She was treated with doxycycline.

Histopathologic findings in human patients. In the 4 patients with fatal HGE, lymphoid depletion with variable degrees of macrophage infiltrates, erythro-leukophagocytosis, and increased numbers of apoptotic cells were observed in the spleen (Figure 1E). An increase in plasma cells was seen in the spleen in 1 patient. The lymph nodes of 3 patients who were autopsied also showed lymphoid depletion, and erythrophagocytosis and foamy macrophage infiltrates were observed in 1 patient each. One pediatric patient with lymphadenopathy, who died after a prolonged course with pancytopenia, initially had significant neutrophil infiltrates and macrophage aggregates (lymphadenitis), and 3 weeks later a marked paracortical hyperplasia was noted. Bone marrow was available for examination in 2 patients who recovered from severe illness associated with pancytopenia; the bone marrow was normocellular and all hematopoietic elements were present in normal numbers. Both bone marrows had evidence of immunologic reactivity with paratrabeular and non-paratrabeular lymphoid aggregates and plasmacytosis. Bone marrow was available for examination in only 2 patients who died after HGE; one patient had prior evidence of chronic lymphocytic leukemia and possible large cell lymphoma. The bone marrow was hypercellular, and although all hematopoietic lineages were present, there was a mild reduction in numbers of mature granulocytes. Other findings included a diffuse increase in foamy histiocytes and slight erythrophagocytic activity. Evidence of leukemia and lymphoma was absent. The second patient was investigated for pancytopenia and the possibility of a myelodysplastic syndrome, but was found to have bone marrow hyperplasia with an atypical lymphoid infiltrate on biopsy. The infiltrate was polymorphous, including both B and T lymphocytes, and could not be definitively classified as malignant.

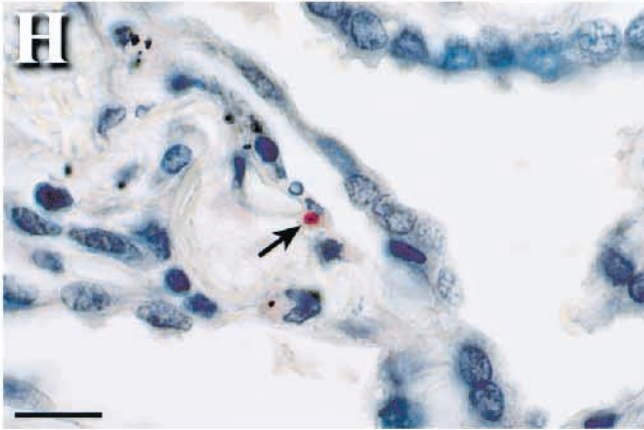
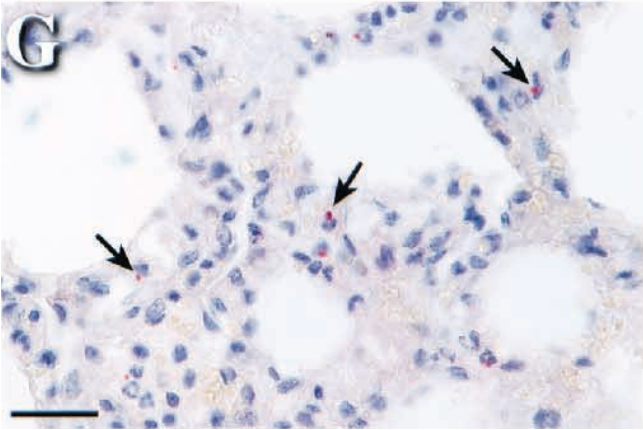
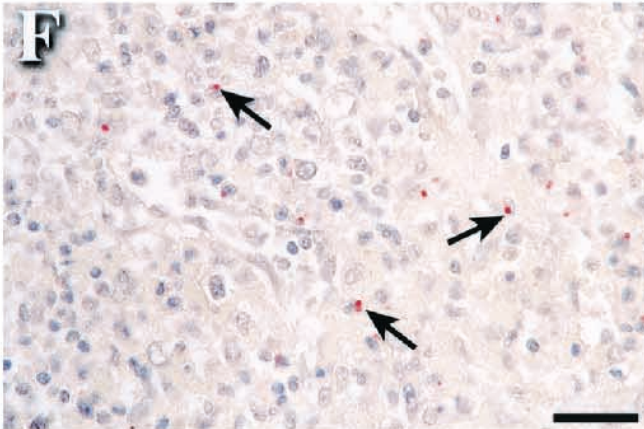
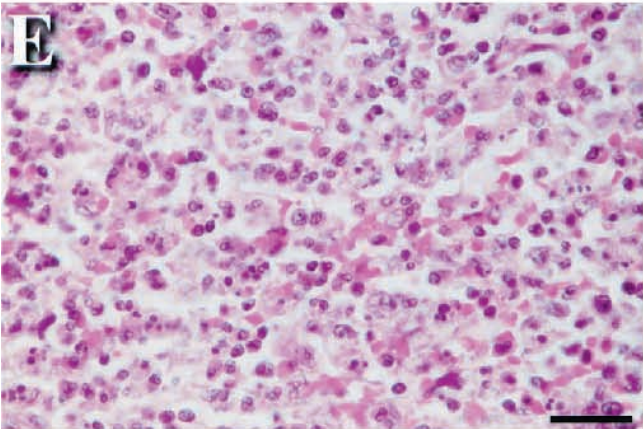
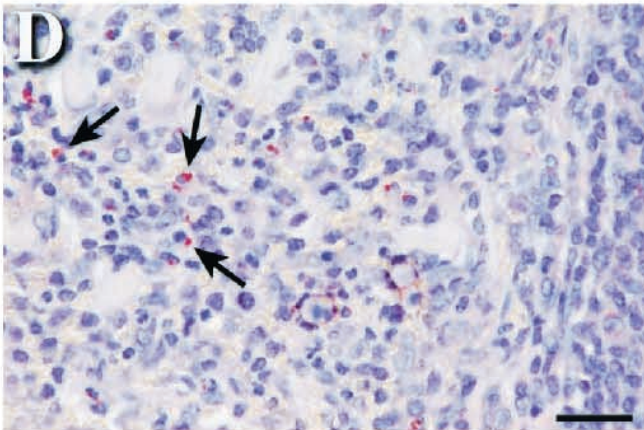
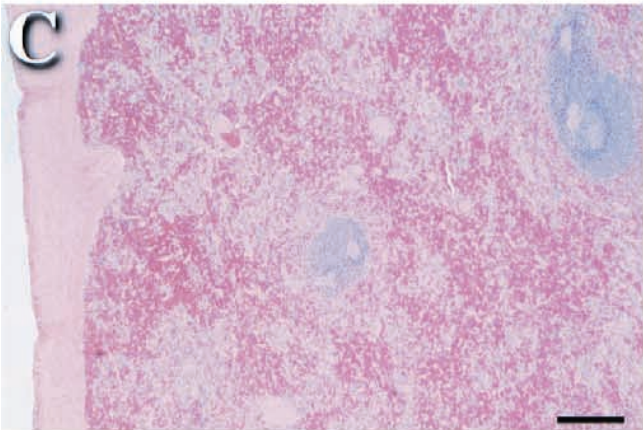
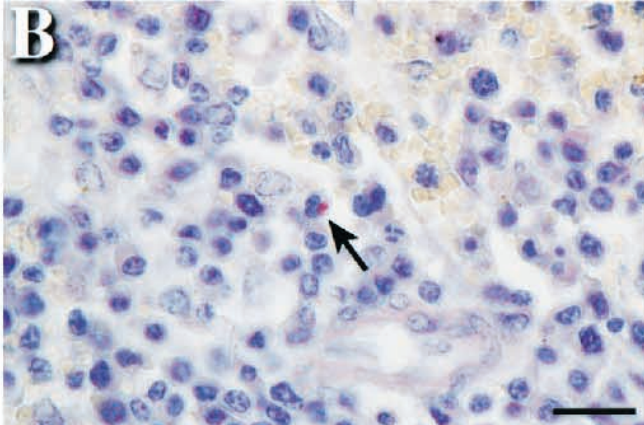
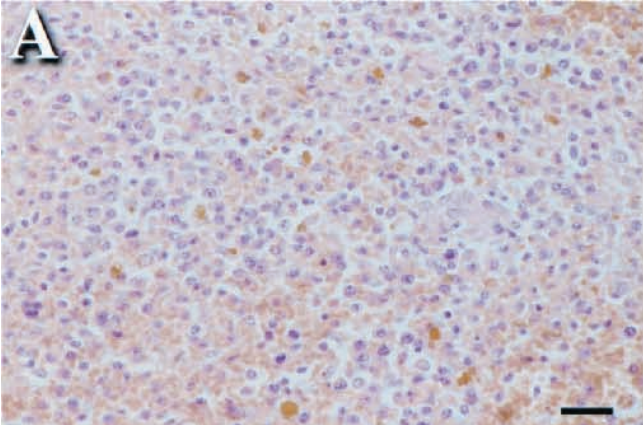
Hepatic pathology was particularly uniform among the 4 fatalities. There were mild lymphohistiocytic periportal infiltrates in all cases, mild to moderate lymphohistiocytic lob-

ular hepatitis with occasional aggregates of lymphocytes, macrophages, and variable numbers of neutrophils. Kupffer cell hyperplasia was observed in 2 cases, and apoptotic hepatocytes associated with minimal or no inflammatory focus in 3 cases (Figure 2C). Inflammatory lung involvement was evident in only 1 case in which mild interstitial lymphohistiocytic infiltrates associated with interstitial edema and intra-alveolar hemorrhage was observed. No evidence of diffuse alveolar damage was found. One patient had overwhelming invasive pulmonary aspergillosis, and underlying pathology attributable to HGE could not be clearly assessed. In 2 additional patients, the cause of death was related to opportunistic infections, including exsanguinating hemorrhages from ulcerative esophagitis due to *Candida* spp. infection in 1 patient and Herpes simplex virus infection in another who had a concurrent focus of *Cryptococcus* spp. pneumonia. The fourth fatality resulted from myocarditis of unknown etiology and a probable cardiac arrhythmia. The cause of death in the pediatric patient is unknown.

Histopathologic findings in experimentally infected horses. After inoculation, the experimentally infected animals developed typical clinical manifestations of equine granulocytic ehrlichiosis,¹⁵ including the presence of fever, decreased appetite and activity, distal limb edema, leukopenia, thrombocytopenia, and laboratory evidence of mild hepatic injury. Examination of the tissues revealed that ehrlichial infection was established in each animal and showed evidence of disseminated disease. The main features were inflammatory lesions in many organs.

The spleens of the *E. equi*-infected horses showed mild lymphoid depletion and erythrocyte congestion in the red pulp with many macrophages, including occasional leukoerythrophagocytic cells in some samples (Figure 1A). In 1 lymph node, early paracortical hyperplasia was detected. Bone marrows were normocellular to hypercellular with normal maturation of all hematopoietic lineages, and granulomas, hemophagocytosis, and plasmacytosis were absent. Pulmonary lesions varied from only mild perivascular lymphohistiocytic infiltrates to patchy, alveolar wall edema and infiltrates of mostly lymphocytes and macrophages. However, 1 horse demonstrated more significant pathologic injury with focal alveolar wall necrosis, intra-alveolar fibrin and inflammatory cell exudates, and interstitial pneumonitis. In the liver, the findings ranged from mild periportal mononuclear cell infiltrates to slight lobular hepatitis with intrasinusoidal aggregates of macrophages and occasional neutrophils, and focal apoptotic cells occasionally associated with small inflammatory lesions (Figure 2A). Vasculitis with intense perivascular infiltrates of mixed inflammation was observed in 2 of 3 horses in which skeletal muscle and tendons of the extremities were examined, but was not detected in any other tissue. The kidney showed dense interstitial infiltrates mainly composed of mononuclear cells and few neutrophils in only 1 animal. The small intestine showed a submucosal hemorrhage with perivascular lymphohistiocytic infiltrates in 1 animal. The examination results of heart, brain, and spinal cord were unremarkable.

Histopathologic findings in an experimentally infected sheep. After inoculation, the experimentally infected lamb developed fever and ehrlichemia. On day 5 (the second day of fever) its temperature was 41.6°C and blood smear count-



ing showed that 56% of the neutrophils contained inclusions (absolute number of infected cells: 0.86×10^9 neutrophils/L). The spleen of the *E. phagocytophila*-infected sheep revealed a slight diminution of lymphoid cells (Figure 1C) with increased numbers of macrophages and neutrophils in splenic sinuses. Paracortical hyperplasia was present in the lymph node with occasional hemophagocytic cells. The liver was characterized by mild periportal lymphocytic infiltration and small aggregates of macrophages with adjacent apoptotic hepatocytes (Figure 2B). The lung contained a mild focal and perivascular mononuclear interstitial infiltrate without necrosis and alveolar damage. No heart, brain, or muscle lesions were observed.

Immunohistologic staining for the *E. phagocytophila* group. Immunostaining revealed that greater than 95% of the infected cells in tissues were mature neutrophils. Characteristic morula forms were observed as intracytoplasmic vacuolar microcolonies of ehrlichiae. Approximately 90% of infected neutrophils present in the organs were seen within vessel lumens, mainly sinusoids of the red pulp in the spleen of 1 human patient, the horses, and the sheep (Figure 1B, D, and F), or in the microvasculature in the lung in a human and a horse (Figure 1G and Table 1). Infected neutrophils were infrequently seen in hepatic sinusoids, bone marrow, kidney glomeruli, heart capillaries, meningeal vessels, cerebral capillaries, adrenal, and rarely in other organs. Rare examples of morulae were detected in mononuclear cells infiltrating the pulmonary alveolar walls in patient 1 (Figure 1H), in mononuclear cells in the myocarditis of patient 4 (26), and in the lymph node of patient 5. No ehrlichiae were observed in lymph nodes of other patients or animals, brain, muscle, spinal cord, or small intestine. However, a small number of mononuclear cells in horse spleen and bone marrow and in sheep spleen contained ehrlichial morulae (Figure 1D). These cells had the morphology of mononuclear phagocytes or other undifferentiated cells.

Infected cells were rarely observed in normal alveolar capillaries. When detected in the lung of a horse, ehrlichial morulae were associated with thickened alveolar walls that contained lymphocytes, macrophages, and infrequent neutrophils, although most of these inflammatory foci lacked infected cells. Although the largest number of infected cells were detected in splenic tissues, the localization of infected cells in spleen was not associated with any specific pathologic change. In the liver, infected cells were as individual infected neutrophils, some apoptotic, that were adherent to sinusoids, and as individual cells associated with small aggregates of mixed inflammatory cells within sinusoids.

Most inflammatory cell aggregates in the other organs lacked infected neutrophils. Morulae were not detected in perivascular inflammatory infiltrates or in foci of vasculitis in tendon or skeletal muscle of the horse extremities.

DISCUSSION

A wide variety of vertebrates, including horses, sheep, goats, cattle, dogs, llamas, and humans, among other animals, can develop granulocytic ehrlichiosis, a mild to severe illness.²⁸ The spectrum of host tissues infected by ehrlichial agents remains under investigation. A few studies have described the microscopic pathologic lesions and imply that the most dramatically involved organs are those of the mononuclear phagocyte system, especially the spleen, liver, bone marrow, and lymph nodes.^{17,22-24} This observation reflects the disseminated distribution of the infectious agent and the systemic immune and inflammatory response of the host.

Among the different mammalian species that were studied here, the most uniform pathologic similarities were also found in the spleen, liver, and lymph nodes. Such findings as mild lymphoid depletion and erythrophagocytosis in the spleen, small sinusoidal and perivenular inflammatory cell aggregates with occasional apoptotic hepatocytes in the liver, and paracortical hyperplasia and histiocytosis in the lymph nodes seem to be common with granulocytic ehrlichiosis regardless of the infected host species. Humans and other mammals infected with ehrlichiae usually lack significant tissue necrosis, abscess formation, or other severe inflammatory reactions. Thrombosis and vasculitis that occur during many rickettsial diseases are usually absent during ehrlichial infection. However, vasculitis was present in horses, but only in skeletal muscle and tendons of the extremities. A proliferative and necrotizing vasculitis of small arteries and veins in the legs was described previously and was implied to be the major underlying pathologic abnormality, a finding not substantiated here.¹⁵ In addition, at least one horse demonstrated pulmonary alveolar damage associated with interstitial pneumonitis, a lesion that suggests the potential for incipient diffuse alveolar damage.

The pathogenesis of ehrlichiosis is poorly understood. Clearly, after entering the dermis via tick bite inoculation and spread, presumably via lymphatics or blood, ehrlichiae invade target cells of the hematopoietic and lymphoreticular systems. The *in vivo* target cells of ehrlichiae are professional phagocytes, granulocytes in cases of *E. phagocytophila*-group infections and macrophages in cases of *E. chaf-*

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FIGURE 1. Splenic and pulmonary histopathologic and immunohistologic findings in *Ehrlichia phagocytophila* group infections of horses (A, B, and G), sheep (C and D), and humans (E, F, and H). Horse spleen with erythrophagocytosis and hemosiderin deposition (A, hematoxylin and eosin stained, bar = 20 μ m) and immunohistologic demonstration of *E. phagocytophila* group ehrlichiae (*E. equi*, arrow in B) in a neutrophil present within a splenic sinusoid (immunoalkaline phosphatase/hematoxylin stained, bar = 100 μ m). Sheep spleen with white pulp lymphoid depletion (hematoxylin and eosin stained, C, bar = 200 μ m) and immunohistologic demonstration of *E. phagocytophila*-group (*E. phagocytophila*, arrows in D) in mature neutrophils and in undifferentiated mononuclear cells in the red pulp (immunoalkaline phosphatase/hematoxylin stained, bar = 40 μ m). Human spleen with increased quantities of mononuclear phagocytes, erythrophagocytosis, apoptoses, nuclear debris (hematoxylin and eosin stained, E, bar = 20 μ m) and immunohistologic demonstration of *E. phagocytophila* group morulae (human granulocytic ehrlichiosis agent, arrows in F) in neutrophils within splenic red pulp sinusoids (immunoalkaline phosphatase/hematoxylin stained, bar = 20 μ m). *Ehrlichia phagocytophila* group morulae (*E. equi*) in neutrophils probably present in interstitial horse pulmonary capillaries (G, arrows, bar = 20 μ m), while H shows an *E. phagocytophila* group morula in a mononuclear cell, probably a histiocyte in the interstitial space of human lung (arrow, immunoalkaline phosphatase/hematoxylin stained, bar = 10 μ m).

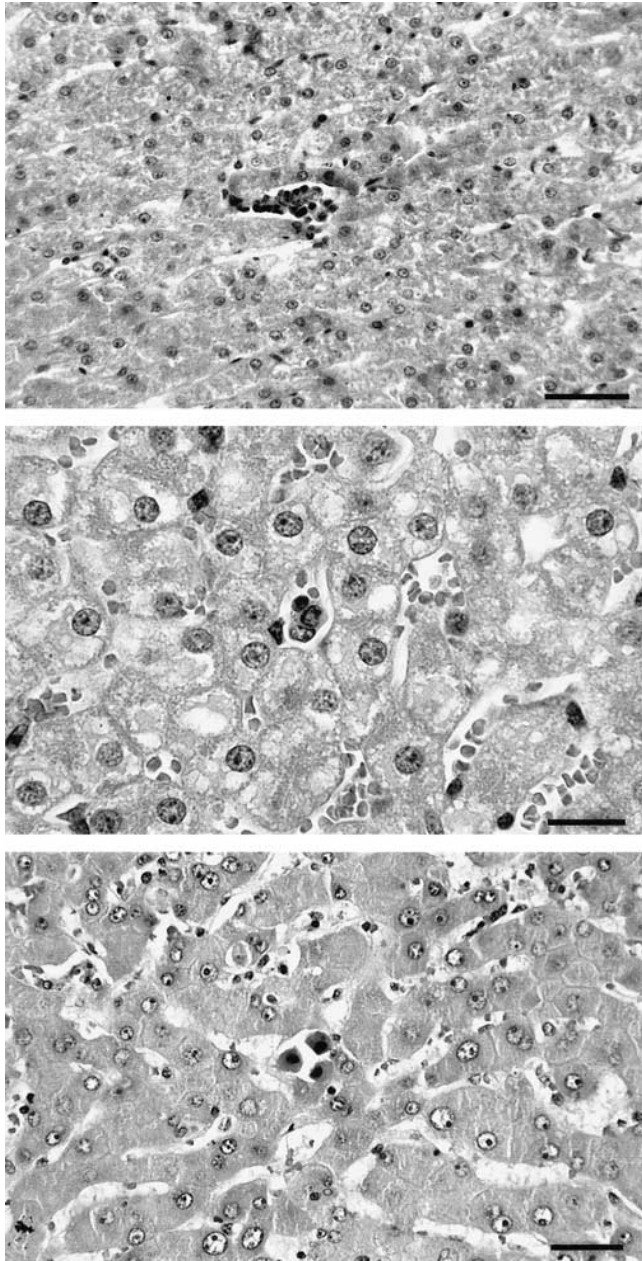


FIGURE 2. Comparison of hepatic histopathology of *E. phagocytophila* group infections in horses, sheep, and humans. Slight lobular hepatitis with focal mononuclear cell aggregate in horse liver (**top**, hematoxylin and eosin stained, bar = 100 μ m) including apoptotic hepatocytes not accompanied by significant inflammation in sheep liver (**middle**, hematoxylin and eosin stained, bar = 20 μ m) and human liver (**bottom**, hematoxylin and eosin stained, bar = 50 μ m).

feensis infection. Ehrlichiae differ from rickettsiae because they replicate within vacuoles of the host cell whereas vasculotropic rickettsiae grow freely within the cytoplasm. Whether or how these granulocytic ehrlichiae directly injure cells is not known despite clear evidence of cytolytic activity *in vitro*.^{25,29} Granulocytic ehrlichiae could initiate a cascade of localized pathologic inflammatory events after invading their hosts. Indeed, neutrophils have been implicated in the pathology of many inflammatory conditions.³⁰ Host tissue

damage may occur through several independent mechanisms. Most importantly, a combination of oxidative and enzymatic processes that appear to be activated simultaneously upon initiation of phagocytosis can lead to significant tissue injury.³¹ However, the triggering of these lytic processes is often accompanied by tissue necrosis at the site of infection. Lesions such as these were rarely observed in this study.

Immunohistologic study has demonstrated that the agents of granulocytic ehrlichiosis are capable of establishing infection in many organs and tissues. Typically, the heaviest burden of infection is seen in the spleen, lungs, and liver. However, in a few cases, focally heavy infection in granulocytes in the kidneys and heart is observed. A large number of infected neutrophils present in the organs were seen within lumens of blood vessels, especially in sinusoids of the red pulp in the spleen or microvasculature in the lung. These infected neutrophils were probably circulating cells as they are also seen in vessels of organs without microscopic damage.

The concurrence of infected neutrophils at sites of early inflammatory aggregates in hepatic sinusoids and in regions of interstitial inflammatory infiltrates in the lungs supports the hypothesis that most pathologic lesions are initially related to focal sequestration of infected cells. The relative lack of infected cells in larger inflammatory lesions further suggests that the inflammatory and immune infiltrates recruited to the site where infected cells adhere are successful in controlling the infection. However, in spite of the relatively minor pathologic findings, a disparity exists between the small number of organisms in tissues and the relatively larger number of pathologic lesions induced, especially in the liver where occasional hepatocytes undergo cell death without evidence for ehrlichial infection or accompanying inflammation. These findings suggest that infected cells that are released into the peripheral circulation adhere to endothelial cell or vascular surfaces, usually in peripheral capillary beds. The presence of ehrlichiae or ehrlichia-mediated modifications to the infected cells induce localized inflammatory responses that may account for tissue damage as reflected by increased serum aminotransferase activities and pulmonary infiltrates in human patients.

Particularly severe consequences of HGE in humans include a toxic or septic shock-like syndrome, occasionally associated with multiorgan failure, and the development of adult respiratory distress syndrome.^{22,24,28,32} While the pathologic correlate of respiratory distress in humans with HGE is uncertain, at least 1 horse developed early lesions suggestive of incipient diffuse alveolar damage, a known pathologic sequelae of systemic inflammatory response due to systemic cytokine release in response to endotoxemia. Similarly, a severe consequence of infection in horses is the development of significant distal limb edema. The pathologic findings here confirm vasculitis or inflammatory endothelial barrier dysfunction in extremity tendons and skeletal muscle in the absence of readily detected ehrlichia-infected cells.

As expected, greater than 95% of the infected cells were mature neutrophils. However, rarely other differentiated cells also were found infected in heavily infected patients and animals, including fibroblasts, endothelial cells, and mononuclear phagocytes. A small number of mononuclear cells

TABLE 1

Summary and comparison of pathologic findings and distribution of *Ehrlichia phagocytophila* group ehrlichiae by immunohistologic methods in experimentally-infected animals and naturally-infected humans*

	Humans	Horses†	Sheep
Spleen	+++ Lymphoid depletion, histiocytic infiltrates, mild erythro-leukophagocytosis	+++ Lymphoid depletion, mild erythro-leukophagocytosis	+++ Lymphoid depletion, histiocyte and neutrophil infiltrates
Lung	+ Mild lymphohistiocytic interstitial pneumonitis	++ Mild perivascular lymphohistiocytic infiltrates to alveolar wall necrosis and interstitial pneumonitis	+ Mild perivascular and interstitial lymphohistiocytic infiltrate
Liver	+ Periportal lymphocytic infiltrates, macrophage aggregates, lobular hepatitis, apoptotic cells	+ Periportal lymphocytic infiltrates, macrophage aggregates, lobular hepatitis, apoptotic cells	- Periportal lymphocytic infiltrates, macrophage aggregates, apoptotic cells
Bone marrow	- Normo- to hyper-cellular ± foamy histiocytes and erythrophagocytosis	ND Normo- to hyper-cellular	ND Not examined
Lymph node	- Lymphoid depletion to paracortical hyperplasia	- Mild paracortical hyperplasia	- Paracortical hyperplasia, erythrophagocytosis
Heart‡	+	+	-
Brain‡	-	-	-
Kidney‡	+	+	+
Muscle‡	-	-	-

* Each immunohistologic feature was scored as few (+), moderate (++), or many (+++). ND = not determined.

† 2 of 3 horses demonstrated intense mixed inflammatory cell vasculitis in extremity soft tissues and muscle. Vasculitis was not identified in any other organ of horses or any tissue of humans or sheep.

‡ No consistent pathologic findings were observed in heart, brain, kidney, or skeletal muscle tissues.

that contain ehrlichiae were identified in bone marrow and spleen, and although these cells may be mononuclear phagocytes, it also possible that they represent progenitor stages of differentiating hematopoietic cells from which the ehrlichia infection is propagated. The etiologic agent of HGE has recently been cultivated in the HL-60 promyelocytic leukemia cell line which has the ability to differentiate along both granulocytic and monocytic cell lines.^{25,33,34} In another recent study, the HGE agent was shown to replicate *in vitro* in immature bone marrow progenitors, granulocytic and monocytic cells, suggesting that both lineages or a common progenitor stage are potential targets of infection *in vivo*.³⁴

Infection of bone marrow progenitors and differentiated circulating cells is unlikely to directly contribute to more severe hematologic manifestations of ehrlichial infections. The presence of normal cellularity or diffuse hyperplasia of bone marrow, combined with the presence of hemophagocytosis in spleen and lymph node, and infected cells in spleen and lung, support peripheral sequestration, consumption, or destruction of normal blood elements as the major mechanism for ehrlichia-induced pancytopenia. The mechanism by which sufficient cells are removed to cause pancytopenia is unknown. The inflammatory lesions precipitated by adherent infected neutrophils are likely to recruit far greater numbers of uninfected blood-derived leukocytes and platelets. However, the presence of minor to significant degrees of leuko-hemophagocytosis in spleen, lymph node, bone marrow, and liver are very likely to also contribute to the diminished leukocyte and platelet blood concentrations that are frequently observed.^{24,35} Ordinarily, a very low burden of circulating infected cells and significant reduction in concentrations of cells that are refractory to infection by *E. phagocytophila*-group ehrlichiae, such as lymphocytes, erythrocytes, and platelets, are observed in HGE. In conjunction with the pathologic observations, these findings

suggest that direct cytopathic injury by ehrlichiae is infrequently a major pathologic mechanism of leukopenia, thrombocytopenia, and anemia.

Because ehrlichiae are difficult to identify in tissues, one might speculate that the pathogenesis of ehrlichiosis is not caused directly by the organism but could be in part host-mediated. Some histologic changes observed in infected animals and humans, such as erythrophagocytosis, could result from aberrant cytokine-mediated stimulation of the host histiocytes. The hepatic pathology includes a variety of lesions ranging from focal hepatic necroses to inflammatory cell aggregates. Moreover, the degree of pulmonary inflammation and alveolar damage, and the presence of extremity vasculitis, both in the absence of significant numbers of ehrlichiae, are not consistent with direct ehrlichia-mediated injury. Rather, these findings suggest the induction of nonspecific mononuclear phagocyte activity and the potential for immunopathologic or cytokine-mediated hepatic injury as potential pathogenetic mechanisms.

Recently, it was shown that neutrophils have the capacity to synthesize and release several cytokines, either constitutively or following stimulation.³⁶ For instance, they are able to secrete pro-inflammatory cytokines like interleukin-1 β or tumor necrosis factor- α and chemokines like IL-8 or macrophage inflammatory protein-1 α . The amount of cytokines secreted by each neutrophil is low but the number of this cell type is greater, both in blood and in inflamed tissues. Macrophage inflammatory protein-1 α or other chemokines may signal mononuclear cell recruitment.³⁷ Thus, the ehrlichia-infected neutrophils could release cytokines inducing the recruitment and activation of mononuclear inflammatory cells, especially mononuclear phagocytes, explaining the presence of hepatic and pulmonary lesions. The HGE agent-infected HL-60 cells produce a variety of chemokines *in vitro*, suggesting a myelosuppressive role for these cytokines

in the generation of pancytopenia.³⁴ This conclusion is inconsistent with the bone marrow hyperplasia and the lack of maturation arrest observed in humans and experimentally infected animals, but otherwise confirms the potential of infected cells to produce cytokines that may allow recruitment of effector cells that could lead to more severe cellular and tissue injury.

Granulocytic ehrlichioses caused by members of the *E. phagocytophila*-group are diseases that trigger dysfunction or suppression of host defenses. It is well established that sheep infected with *E. phagocytophila* are predisposed, like humans during HGE and horses infected with *E. equi*, to develop opportunistic infections and secondary infections with bacteria, fungi, and viruses.^{1,28,38,39} These animals develop defects in both humoral and T cell-mediated immunity and abnormalities in normal neutrophil phagocytic and migratory functions. In fatal human cases, patients died after *Candida* esophagitis, Herpes simplex virus esophagitis, cryptococcal pneumonitis, and invasive pulmonary aspergillosis.^{5,22,23} Neutrophil dysfunction in granulocytic ehrlichiosis is not unexpected because neutrophils represent the host cells of these bacteria. Neutrophils are essential for host defenses since they are the first cells to be recruited to sites of infection or injury. Neutrophil dysfunction, whether hyper-responsive or hypo-responsive, could result in an immunodepressed state, or in a pro-inflammatory condition such as septic- or toxic shock-like syndrome, or in diffuse alveolar damage leading to adult respiratory distress syndrome that apparently is one severe complication of HGE and human monocytic ehrlichiosis.^{5,17,24,32}

Strong similarities among the pathologic findings and immunohistologic distribution of ehrlichiae in humans and animals with clinical illness due to *E. phagocytophila*-group infections exist. Such findings suggest that considerable progress in understanding the pathogenesis of HGE can be derived from study of *E. equi* infection of horses or *E. phagocytophila* infection of ruminants. These examinations most strongly support an initiating role for infected neutrophils adherent to endothelium in tissues, but further suggest that subsequent tissue injury is mediated locally by accumulating inflammatory cells and systematically by induction of pro-inflammatory responses. Significant additional investigations will be required to elucidate the role of the spleen and bone marrow as sites of ehrlichial replication and the potential role and effects of cytokines released by the infected host in the pathogenesis of *E. phagocytophila*-group infections.

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