Animal Model

Animal Model of Fatal Human Monocytotropic Ehrlichiosis

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Human monocytotropic ehrlichiosis caused by *Ehrlichia chaffeensis* is a life-threatening, tick-borne, emerging infectious disease for which no satisfactory animal model has been developed. Strain HF565, an ehrlichial organism closely related to *E. chaffeensis* isolated from *Ixodes ovatus* ticks in Japan, causes fatal infection of mice. C57BL/6 mice became ill on day 7 after inoculation and died on day 9. The liver revealed confluent necrosis, ballooning cell injury, apoptosis, poorly formed granulomas, Kupffer cell hyperplasia, erythrophagocytosis, and microvesicular fatty metamorphosis. The other significant histological findings consisted of marked expansion of the marginal zone and infiltration of the red pulp of the spleen by macrophages, interstitial pneumonitis, and increased numbers of immature myeloid cells and areas of necrosis in the bone marrow. Ehrlichiae were detected by immunohistology and electron microscopy in the liver, lungs, and spleen. The main target cells were macrophages, including Kupffer cells, hepatocytes, and endothelial cells. Apoptosis was detected in Kupffer cells, hepatocytes, and macrophages in the lungs and spleen. This tropism for macrophages and the pathological lesions closely resemble those of human monocytotropic ehrlichiosis for which it is a promising model for investigation of immunity and pathogenesis. (*Am J Pathol* 2001, 158:757-769)

Ehrlichiae are obligately intracellular gram-negative bacteria that reside within a cytoplasmic vacuole of the host cell. These bacteria have evolved in close association with a tick vector and a zoonotic host.¹⁻³ In 1987 the first case of human monocytotropic ehrlichiosis (HME) was reported in the United States,⁴ and subsequently in 1991 the etiological agent of HME, *Ehrlichia chaffeensis*, was

isolated and characterized through analysis of the 16S rRNA gene sequence.^{5,6}

HME is a multisystemic disease with clinical manifestations that range from mild to severe illness, and a mortality rate of 3 to 4% despite the excellent efficacy of doxycycline treatment.⁷⁻¹¹ HME can affect previously healthy, immunocompetent patients or severely immunocompromised patients (eg, acquired immune deficiency syndrome) in whom the infection is often overwhelming.¹² More than 740 cases and 1,500 cases have been confirmed at the Centers for Disease Control and Prevention and at a large reference laboratory (MRL Diagnostics, Cypress, CA), respectively, totaling more than 2,200 cases of HME diagnosed in 47 states.^{11,13} The epidemiological importance of this disease is highlighted by an on-going prospective study in Cape Girardeau in southeastern Missouri, where the provisional incidence was 11 cases per 100,000 population in 1998.¹¹ This figure is most likely an underestimate because one primary care physician's office practice accounted for the majority of cases. Other studies have shown that HME is even more prevalent than Rocky Mountain spotted fever in states such as North Carolina where Rocky Mountain spotted fever is highly endemic.¹⁴

The currently available animal models of *E. chaffeensis* infection are unsatisfactory. Several reports of inoculation of *E. chaffeensis* into immunocompetent mice, ie, BALB/c, C3H/HeJ, C.B-17, C3H/HeN, DBA, and C57BL/6 have shown resistance to the development of disease.¹⁵⁻¹⁹ The presence of the organism in the tissues and blood after inoculation was detected only by polymerase chain reaction amplification of ehrlichial DNA genes after inoculation. Seroconversion occurs several days after inoculation of the animals with *E. chaffeensis*, but few of the animals develop any illness, and none die. Furthermore, the animals do not develop histopathological lesions mimicking the disease in humans. For the above reasons,

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these animal models are inadequate for the study of HME. The inoculation of *E. chaffeensis* into immunocompromised mice, ie, SCID mice, produces severe disease with fatal outcome.^{18,19} However, this model is far from ideal because of development of histopathological lesions that do not resemble the lesions observed in humans. Furthermore, the use of immunocompromised animals is a very unrealistic model to study the evolution and development of the immunological events of the disease.

Alternatively, the infection caused by *Ehrlichia muris*, an ehrlichia genetically closely related to *E. chaffeensis*, in immunocompetent mice, ie, BALB/c mice, causes disseminated infection associated with mild disease and minimal mortality.²⁰ The absence of death or severe disease are major drawbacks of this model for investigating pathogenesis and protective immunity.

Ehrlichia canis infection causes disease in dogs with histopathological lesions during the acute stage similar to those of HME.^{21–24} However, the absence of inbred syngeneic dogs, including animals with genetically defined immune defects, the lack of data on the sequence of events during the host defense responses in dogs, and the lack of commercially available canine-specific markers for immune effectors are significant limitations of this model.

Thus, the need for a better animal model to study HME is of utmost importance. The ideal animal model would use *E. chaffeensis* or a closely related ehrlichia with the capacity to produce severe disease and death in immunocompetent animals. The histopathological lesions should be similar to the lesions observed in HME, and the severity of disease and lethality should be predictable based on the dose of inoculum.

In the present study, we report the histopathological findings of an excellent animal model for HME. C57BL/6 mice were inoculated with an ehrlichial organism [*Ixodes ovatus* ehrlichia (IOE), strain HF565] isolated from *I. ovatus* ticks in Japan.²⁵ IOE is closely related genetically to *E. chaffeensis* by 16S rRNA sequence analysis.²⁶ It has also been demonstrated that the sequences of the p28 multigene family of IOE are closely related to *E. chaffeensis*, *E. muris*, and *E. canis* (JW McBride and X-J Yu, unpublished data). Furthermore, there is a close antigenic relationship of the *E. chaffeensis* p28 and cell wall components of IOE as demonstrated by immuno-ultrastructural studies of the reactivity of monoclonal antibodies to *E. chaffeensis* p28 against the outer aspect of the cell wall of IOE (JW McBride and VL Popov, unpublished data).

Materials and Methods

Ehrlichia

Liver and spleen harvested from ddY mice inoculated with IOE were received frozen on dry-ice as a generous gift from Dr. Makoto Kawahara, Microbiology Department, Nagoya City Public Health Research Institute, Nagoya, Japan. The organs were weighed and ground *in toto* at a 10% w/v concentration in sucrose-phosphate-

glutamate buffer (0.218 mol/L sucrose, 0.0038 mol/L KH_2PO_4 , 0.0072 mol/L K_2HPO_4 , 0.0049 mol/L monosodium glutamic acid, pH 7.0). To produce ehrlichial stocks for reproducible studies throughout a long period of investigation, 10 C57BL/6 mice were inoculated intraperitoneally with 1 ml of the suspension. On days 10 and 11 after inoculation, the mice were sacrificed, and the spleens and livers were harvested. The organs were then weighed, and a 10% w/v suspension was prepared by homogenizing the organs in sucrose-phosphate-glutamate buffer. Large particles of debris were removed by centrifugation at $190 \times g$ for 3 minutes. The supernatant was then aliquoted and stored at -80°C as 10^{-1} stock of IOE. Quantitation of the organisms was not performed because of the lack of a precise method to do so. However, the LD_{50} of this stock was approximately a dilution of 10^{-5} , because a 10^{-4} dilution killed 100% of the mice and 100% of the mice survived when inoculated with 10^{-6} of the same stock (unpublished data).

Mice

Ten C57BL/6 inbred mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were male and 6 to 8 weeks old.

Infection of Mice

Eight mice were inoculated intraperitoneally with 1 ml of a 10^{-4} dilution of the IOE stock suspension. Two control animals were inoculated intraperitoneally with 1 ml of a 10^{-4} dilution of a homogenate of uninfected spleen and liver. Two infected mice were sacrificed on each of days 2, 5, 7, and 9 after inoculation. The two control mice were sacrificed on day 10 after inoculation. A complete necropsy was performed on each of the animals.

Histology and Immunohistochemistry

The liver, spleen, bone marrow, kidneys, testis, skeletal muscle, and lungs were fixed in 4% neutral-buffered formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin. Paraffin-embedded sections of the liver from two mice sacrificed on day 9 after inoculation were stained by Masson's trichrome and reticulin methods.

Tissue sections of liver, spleen, bone marrow, and lungs from all of the animals were stained immunohistochemically using an automated Ventana ES Immunostainer (Ventana Medical Systems, Tucson, AZ). Briefly, the sections were deparaffinized by heating the glass slides at 70°C for 20 minutes, followed by immersion in three xylene baths for 5 minutes each. The slides were then rehydrated by immersion in a series of alcohol baths ranging from 100 to 80% for 3 minutes each. The slides were then washed in distilled water and placed in Ventana Medical Systems wash solution until they were loaded on the instrument for staining. The slides were then incubated with an endogenous peroxidase inhibitor solution containing hydrogen peroxide and sodium azide

(Ventana Medical Systems) for 4 minutes at 37°C followed by incubation with anti-*E. chaffeensis* rabbit polyclonal antibody for 32 minutes at 37°C. The slides were then washed in phosphate-buffered saline (PBS) and incubated for 8 minutes with a goat biotinylated anti-rabbit IgG (H + L) antibody (Vector Laboratories, Burlingame, CA). The slides were then washed and incubated with avidin-horseradish peroxidase conjugate for 8 minutes at 37°C, followed by incubation with a substrate solution containing 3-amino-9-ethylcarbazole for 8 minutes at 37°C. Counterstaining with hematoxylin was then performed, and the slides were coverslipped. Normal rabbit serum was used as primary antibody as a negative control.

Electron Microscopy

One cubic millimeter pieces of liver, spleen, and lungs were obtained from all experimental animals and fixed in a mixture of 1.25% formaldehyde, 2.5% glutaraldehyde, and 0.03% trinitrophenol in 0.05 mol/L cacodylate buffer, postfixed in 1% osmium tetroxide in the same buffer, stained *en bloc* in 1% uranyl acetate in 0.1 mol/L maleate buffer, and processed further as described previously,²⁷ until embedded in Poly/Bed 812 (Polysciences Inc., Warrington, PA). Ultrathin sections were cut with a Reichert Ultracut S ultramicrotome (Leica Instruments, Deerfield, IL), stained with aqueous uranyl acetate and lead citrate and examined in a Philips CM100 electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) at 60 kV.

Terminal dUTP Nick-End Labeling (TUNEL) Stain

The tissue sections were deparaffinized as described above. The tissue was then permeabilized by covering the entire specimen with a 20 µg/ml solution of proteinase K diluted in 10 mmol/L Tris, pH 8, and incubated for 20 minutes at room temperature. The tissue sections were then washed in Tris-buffered saline (20 mmol/L Tris, 140 mmol/L NaCl, pH 7.6). Inactivation of endogenous peroxidases was accomplished by immersing the tissue sections in 3% hydrogen peroxide diluted in methanol for 5 minutes at room temperature. The glass slides were then placed in equilibration buffer (1 mol/L sodium cacodylate, 0.15 mol/L Tris, 1.5 mg/ml bovine serum albumin, 3.75 mmol/L CaCl₂, pH 6.6) for 30 minutes. The tissue sections were then incubated with 60 µl of a solution containing terminal deoxynucleotidyl transferase and a mixture of biotinylated dNTPs, according to the manufacturer's instructions (terminal deoxynucleotidyl transferase-Fragel; Oncogene Research Products, Cambridge, MA) at 37°C for 90 minutes in a hybridization chamber (Hybri-Well; Sigma Chemical Co., St. Louis, MO). The enzymatic reaction was stopped by incubation with 0.5 mol/L ethylenediaminetetraacetic acid, pH 8, for 5 minutes at room temperature. The slides were then washed with Tris-buffered saline and immersed in blocking buffer for 20 minutes (4% bovine serum albumin in

PBS) followed by incubation with 100 µl of a solution containing peroxidase-streptavidin for 30 minutes at room temperature in a humidified chamber according to the manufacturer's instructions (Oncogene Research Products). The tissue sections were then washed in Tris-buffered saline and covered with a solution containing 3,3'-diaminobenzidine (0.7 mg/ml), hydrogen peroxide and urea (0.6 mg/ml). The slides were then rinsed with distilled water and counterstained with Azure A for 10 seconds. Positive controls were generated by digesting the tissue sections with DNase I (10 µg/ml final concentration) in Tris-buffered saline containing 1 mmol/L MgCl₂ for 20 minutes. Terminal deoxynucleotidyl transferase was omitted in slides used as negative controls. A different set of slides from the same animals was also double-stained by the TUNEL method for apoptosis and by immunohistochemistry with the ehrlichial antibodies described earlier. However, detection of the ehrlichial organisms was performed with alkaline-phosphatase-tagged secondary antibodies (Vector Laboratories, Inc.) and Fast Red as a substrate for color development for optimal distinguishing of the ehrlichial organisms and apoptosis.

Blood Counts

Blood samples from infected and control mice were processed through a Sysmex SE-9000 TOA Medical Electronics Co., Ltd. (Kobe, Japan) automated analyzer for total and differential white blood cell counts, red blood cell counts, and platelet counts.

Serum Evaluation of Hepatic Injury and Function

Serum samples from infected mice on days 2, 5, 7, and 9 and uninfected control animals were assayed in a Vitros Chemistry System 950 Johnson and Johnson Clinical Diagnostics (Rochester, NY) automated analyzer for the hepatic transaminases, alanine and aspartate aminotransaminases, alkaline phosphatase, and total bilirubin concentrations.

Antibody Responses

Serum samples from infected and control mice were measured by indirect immunofluorescence assay using *E. muris* as a surrogate antigen. Antigen slides were prepared as follows: monolayers of DH82 cells were grown in 150 cm² flasks in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. Infection of the monolayer was monitored by Diff Quik staining until 80% of the cells were infected. The cells were harvested, and antigen slides were prepared. Serum samples were serially diluted twofold and incubated with the antigen slides for 30 minutes at 37°C in a humidified chamber. The antigen slides were then washed three times in PBS, pH 7.4, and then incubated with fluorescein isothiocyanate-labeled anti-mouse immunoglobulins (Kirkegaard and Perry, Gaithersburg, MD) at a 1:100 dilution. The slides were then washed three times in PBS, pH 7.4,

counterstained with Evans blue and examined under a Nikon Labphoto fluorescent microscope (Nikon Co., Tokyo, Japan). Serological titers were expressed as the reciprocal of the highest dilution at which specific fluorescence was detected.

Results

Course of Disease

All animals developed severe sickness with ruffled fur, inactivity, and huddling together on day 7 and died on day 9.

Histopathological Observations

The liver, spleen, bone marrow, and lungs were evaluated in detail because these were the organs that showed the most consistent and remarkable changes.

Moderate diffuse ballooning cell injury of hepatocytes and mild Kupffer cell hyperplasia were observed on day 2. Occasional focal collections of mostly neutrophils and scattered macrophages, which were present mostly in the midzonal area of the hepatic lobule, were identified in mice inoculated with either uninfected or infected liver-spleen homogenate. Thus they were not considered to be related to the ehrlichial infection. On day 5, occasional apparent apoptotic bodies were observed associated with focal accumulations of macrophages, and diffuse ballooning cell injury of hepatocytes persisted. On day 7, the Kupffer cell hyperplasia had increased. The macrophage-rich inflammatory infiltrates were more cellular and were consistent with poorly formed granulomas (Figure 1A). Apoptotic cells were more numerous throughout the liver including many that were adjacent to the poorly formed granulomas. The ballooning cell injury of the hepatocytes was notably reduced. On day 9, extensive partially confluent foci of necrosis of contiguous hepatocytes were observed, predominantly in the midzone (Figure 1B). The sinusoidal spaces in the areas of necrosis were widened and formed a peliosis-like configuration but retained the sinusoidal reticulin lining as demonstrated by reticulin and trichrome stains. Scattered neutrophils were observed in the necrotic areas. In the non-necrotic areas, the hepatocytes showed diffuse microvesicular fatty metamorphosis (Figure 1C) as well as occasional, randomly distributed middle-sized droplet fatty change. Occasionally, prominent large vacuoles were observed in Kupffer cells with displacement of the nucleus that gave the cells a signet-ring appearance. Erythrophagocytosis was observed diffusely.

On day 2, the microscopic appearance of the spleen of the experimental and control animals was similar. The

Figure 1. Hepatic histopathology of IOE infection in C57BL/6 mice. On day 7, occasional accumulations of macrophages (arrow) were seen in the hepatic lobules. Ballooning degeneration of hepatocytes was also present (lower right) (A, H&E; original magnification, $\times 200$). On day 9, confluent foci of necrosis were present especially in the midzone (B, H&E; original magnification, $\times 100$). Also on day 9, prominent microvesicular fatty metamorphosis was present in hepatocytes (C, H&E; original magnification, $\times 400$).

Figure 2. Transition between mantle zone of splenic lymphoid follicle (top) and marginal zone was rich in macrophages, immunoblasts, and scattered lymphocytes (H&E; original magnification, $\times 400$).

white pulp showed secondary follicles, and the red pulp showed congested sinusoids with normal foci of hematopoiesis. The mantle zone was conspicuous, but the outer marginal zone was very thin and not readily distinguishable. On day 5, a relative increase in lymphocytes was observed inside the red pulp sinusoids, and the marginal zone was slightly prominent. On day 7, the germinal centers of the secondary follicles showed a starry sky configuration with small quantities of tingible body macrophages and immunoblasts. The marginal zone was prominent and composed of activated, moderately enlarged lymphocytes and macrophages (Figure 2). The red pulp contained focal areas with moderately increased numbers of macrophages and plasma cells. On day 9, the germinal centers contained numerous, prominent tingible body macrophages, and the marginal zone was more expanded with further thinning of the mantle zone. The red pulp was also infiltrated by macrophages.

The bone marrow on day 2 was similar histologically in experimentally infected and control animals. The bone marrow cellularity was 80 to 90%. The myeloid:erythroid (M:E) ratio ranged from 2:1 to 3:1, with normal maturation patterns. Megakaryocytes ranged from 6 to 8 per high-power field (HPF). On day 5, an increase in the M:E ratio was observed with an increase in immature myeloid cells and a decrease in mature myeloid forms. On day 7, there was an even higher proportion of immature myeloid forms, and on day 9 there was striking confluent necrosis, apoptotic marrow cells, and infiltration by neutrophils (Figure 3). In nonnecrotic areas, the M:E ratio was still elevated with predominance of immature myeloid forms.

Figure 3. Bone marrow histopathology of IOE infection in C57BL/6 mice. Extensive areas of confluent necrosis in the bone marrow were observed on day 9. Note abundant karyorrhexis. H&E; original magnification, $\times 200$.

The first significant histopathological change in the lungs was observed on day 7, consisting of moderate monocyte margination in the pulmonary veins. On day 9, the interstitium was multifocally widened and infiltrated by mononuclear cells (Figure 4).

The brain did not show any abnormalities except for the presence of monocyte margination in a meningeal vessel in one animal on day 7. The rest of the tissues did not show any significant histological abnormalities.

Immunolocalization of IOE with Anti-E. chaffeensis Polyclonal Antibody

On days 2 and 5, no organisms were detected immunohistochemically in any of the organs. On days 7 and 9, the liver showed morulae located mostly in cells lining the sinusoidal spaces consistent with Kupffer cells and/or endothelial cells and in macrophages in the sinusoidal lumen (Figure 5A). Hepatocytes also contained organisms although less frequently than the sinusoidal cells. On day 9, the ehrlichial inclusions were more abundant than on day 7. The organisms were not observed in the areas of hepatic necrosis.

The spleen contained ehrlichial morulae in the mononuclear cells in the expanded marginal zones and in the red pulp on days 7 and 9. No morulae were observed in the lymphoid follicles. The lungs contained scattered organisms that were in cells in the interstitium of the alveolar septa (Figure 5B). The bone marrow contained scattered mononuclear cells with ehrlichial inclusions that were most abundant on day 9. The relative ehrlichial burden was, as judged by immunohistochemistry, greatest in the spleen, followed in descending order by liver, lungs, and bone marrow.

Figure 4. Lung histopathology of IOE infection in C57BL/6 mice. On day 9 the interstitium was multifocally widened and infiltrated by mononuclear cells (H&E; original magnification, ×400).

Evaluation of Apoptosis by the TUNEL Method

On day 2, minimal (less than 1 to 2 per 20 HPF) apoptotic events were detected in spleen, lungs, liver, and bone marrow. On day 5, the number of apoptotic events rose to 4 to 5 cells per 20 HPF in the bone marrow. The apoptotic cells were scattered throughout the marrow involving no particular cell type. In the liver, the number of apoptotic events was 2 to 3 per 20 HPF, and the apoptotic cells were mostly located in the sinusoids or lining these structures. No apoptotic hepatocytes were noted at this time point. In the spleen, the number of apoptotic events was 20 to 25 per 20 HPF. The apoptotic cells were present in the germinal centers and the marginal zone. In the lungs the number of apoptotic events was less than 1 per 20 HPF.

On day 7, the liver contained 12 apoptotic cells per 20 HPF, and most of the events were located in the cells lining the sinusoids (Figure 6A). Scattered apoptotic hepatocytes were also present (Figure 6A). The foci of hepatocellular necrosis were indeed not apoptotic. In the lungs and bone marrow, the quantity of apoptotic cells was 0 to 1 per 20 HPF, which was similar to day 5. However, in the spleen the quantity of apoptotic cells increased to 30 to 40 per 20 HPF (Figure 6B). The location of apoptotic cells was similar to that on day 5.

Figure 5. IOE immunolocalization with anti-*E. chaffeensis* polyclonal antibody. **A:** Liver with presence of ehrlichial organisms in hepatocytes (arrow), macrophages in the lumen of sinusoids, and Kupffer cells. **B:** Lungs with ehrlichial organisms in the alveolar septa. Horseradish peroxidase (diaminobenzidine)/hematoxylin stain. Original magnification, ×400.

Figure 6. Demonstration of apoptotic bodies by TUNEL stain. **A:** Liver section showing an apoptotic hepatocyte (arrow) and an apoptotic cell lining a hepatic sinusoid (arrowhead). **B:** Splenic, marginal zone showing multiple apoptotic bodies (arrows). TUNEL technique, horseradish peroxidase (diaminobenzidine)/Azure A stain. Original magnification, $\times 400$.

On day 9 the spleen showed a similar high quantity and distribution of apoptosis as on day 7. The liver contained its highest quantity of apoptotic events (20 to 30 per 20 HPF), involving the same distribution. At this time,

a higher portion of apoptotic hepatocytes was noted. In the lungs the frequency of apoptosis also rose to 4 to 5 per 20 HPF, whereas the bone marrow showed extensive areas of necrosis that did not contain apoptotic cells. A few cells surrounding the necrotic areas were apoptotic. The uninfected control animals did not show any apoptotic events in liver, bone marrow, or lungs. Double-staining of the TUNEL slides with anti-*E. chaffeensis* antibodies revealed absence of apoptosis in the infected cells. Furthermore, clearly apoptotic cells did not contain cytoplasmic ehrlichial morulae.

Ultrastructural Observations

Ehrlichial inclusions (morulae) in different stages of development were observed in spleen, liver, and lungs on days 7 and 9. On day 7 ehrlichiae were localized mostly in perisinusoidal macrophages in the spleen. Large host cell vacuoles or morulae (3 to 5 μm in diameter), predominantly filled with dense-cored ehrlichiae suspended in dense intramolecular matrix, were present in the cytoplasm of these cells (Figure 7). Rarely, macrophages were observed with smaller morulae containing few large reticulate ehrlichial cells. Some of the macrophages infected with ehrlichiae contained apoptotic bodies (fragments of other cells), phagocytized red blood cells, other phagolysosomes, and short cisterns of granular endoplasmic reticulum (Figure 7, A and B). Elongated dense-cored ehrlichiae showed constrictions in their central region, suggesting that they were undergoing binary fission. On day 9 infected macrophages revealed nuclear condensation consistent with the initial stages of apoptosis (Figure 7C).

In the liver, ehrlichiae were found mainly in Kupffer cells, but they were also detected in hepatocytes (Figure 8, A and B). In Kupffer cells, large morulae were observed filled either with dense-cored ehrlichiae or with reticulate cells embedded in a fibrillary matrix. Often the nucleus of the host cell was compressed by the morula and had a semilunar shape. On day 9 a central hepatic vein was observed in which virtually all of the endothelial cells harbored ehrlichial morulae (Figure 8C). Ehrlichial morulae composed mostly of reticulate cells were identified in hepatocytes (Figure 8B). On day 9 hepatocytes also contained numerous lipid droplets. Some hepatocytes showed large vacuoles with fibrillary contents that displaced the nucleus to the margin of the cell, resembling a signet ring.

In the lungs, numerous morulae were present in alveolar endothelial cells on day 9 (Figure 9, A and B). Ehrlichial morulae were also observed in interstitial macrophages. Ehrlichial morulae were also found in neutrophils in the spleen and in eosinophils in the lung, but they were smaller and contained fewer ehrlichiae than those in macrophages.

Blood Counts

The number of platelets and white blood cells decreased significantly during days 7 and 9 after infection (Table 1).

Figure 7. Ultrastructure of spleen in C57BL/6 mice 7 days after infection with ehrlichiae isolated from IOE tick (strain HF 565). Scale bar, 1 μ m. **A:** A sinusoidal-lining macrophage containing large morula limited by a membrane (arrow). **B:** Portion of a splenic macrophage with two ehrlichial morulae. IOE in the morulae are suspended in a fibrillar matrix (f). Some ehrlichiae have constrictions in their middle suggesting binary fission (arrows). The macrophage (N = its nucleus) contains a phagocytosed platelet (p), an erythrocyte (e), and an apoptotic body (a). **C:** IOE-infected splenic macrophage (9 days after infection) with appearance of apoptosis. E, reticulate ehrlichiae inside a morula. Arrows indicate condensed nuclear chromatin characteristic of apoptosis.

The red blood cell mass did not reveal any significant differences during the experimental intervals or with respect to the control animals.

Hepatic Tests

One animal from each experimental group was evaluated. Aspartate aminotransaminase levels rose during days 7 and 9 after inoculation (712 and 816 IU/dl, respectively), and the elevation of alanine aminotransaminase levels was less pronounced (Table 2). Both alkaline phosphatase and bilirubin concentrations were within the normal range.

Antibody Responses

The serological titers on day 2 remained $<1:20$. On days 5, 7, and 9, the titers were elevated at 1:320, 1:160, and 1:80, respectively.

Discussion

Fourteen years have elapsed since the first case of HME was described.⁴ However, to date no adequate animal models exist for the study of this potentially fatal tick-borne disease. Previous attempts to infect mice with *E. chaffeensis* have yielded discouraging results.^{15–19} The ehrlichial organism used in our model is closely related to *E. chaffeensis* based on sequence analysis of the 16S rRNA subunit gene and the GroEL gene. The histopathology of the lethal infection of C57BL/6 mice with IOE strain HF565 isolated from *I. ovatus* ticks in Japan resembles autopsy findings in fatal HME of immunocompetent patients, including hepatic apoptosis, erythrophagocytosis, interstitial pneumonia, and myeloid hyperplasia of the bone marrow, and the tropism of the ehrlichia for mononuclear phagocytes mimics that of *E. chaffeensis* in HME.

Although humans have been documented to become infected with at least four species of Ehrlichia, namely *E. chaffeensis*, *E. phagocytophila*, *E. ewingii*, and *E. canis*^{8,9,11,13,18,28,29}, some persons portray the diseases as essentially the same. This concept is not true, and some of the conclusions are based on inappropriately interpreted serological data. Although the severity of the different ehrlichioses ranges from asymptomatic to fatal, it should be recognized that infection with *E. chaffeensis* is frequently severe, potentially fatal, even in immunocompetent persons, and unlikely ever asymptomatic. In contrast, *Ehrlichia canis* has been isolated from the blood of a healthy person.³⁰ *Ehrlichia ewingii* has been associated

Figure 8. Ultrastructural features of the liver of C57BL/6 mice infected with IOE. Scale bar, 1 μ m. **A:** Ehrlichial morulae in two Kupffer cells (arrows) on 7 day after infection are large and displace and compress the host cell nuclei. **B:** Small morula containing reticulate cells of IOE (arrow) in the cytoplasm of a hepatocyte 9 days after infection. The hepatocyte has numerous (13 in this portion of the section) lipid droplets (d). **C:** Ehrlichial morula in an endothelial cell lining a central vein of hepatic lobule, 9 days after infection. L, lumen of the vein. Arrow indicates an intercellular junction between endothelial cells. N, nucleus of an adjacent hepatocyte. The morula contains both reticulate cells (r) and dense-cored cells (d).

Figure 9. IOE in the lungs of C57BL/6 mice 9 days after infection. **A:** Alveolar septal endothelial cell contains a large morula of IOE with reticulate cells (**thick arrow**). The endothelial cell has a characteristic intercellular junction (**thin arrow**) and abundant micropinocytotic vesicles (**arrowhead**) at its surface adjacent to the basement membrane. **B:** Morulae containing either reticulate (r) or dense-cored ehrlichiae (d) in cells within the alveolar septal wall. A morula with dense-cored cells has abundant fibrillar matrix (f). AS, alveolar space. **Arrow** indicates a type I alveolar lining cell.

with illness principally in immunocompromised persons.²⁹ Asymptomatic development of antibodies reactive with *E. chaffeensis* occurred in soldiers heavily exposed to the species of tick, *Amblyomma americanum*, that also is considered to be the most likely carrier of *E. ewingii* and an unnamed *Ehrlichia* species that is highly prevalent in white-tailed deer.^{31–33} It is very likely that antibodies stimulated by these ehrlichiae cross-react with *E. chaffeensis*. The ehrlichial organism that has received the most attention, the agent of human granulocytotropic ehrlichiosis or *E. phagocytophila*, does not often cause

illness as severe as HME, which is often comparable in severity to Rocky Mountain spotted fever and toxic shock syndrome.^{4,8,13,34} HME is well recognized to be associated with life-threatening adult respiratory distress syndrome and meningoencephalitis even in immunocompetent patients.^{35–39}

Pulmonary manifestations of HME include cough, dyspnea, tachypnea, and pleural effusions.^{7,35,39,40} Chest radiographs have shown findings that range from pulmonary infiltrates to those characteristic of the clinical condition, adult respiratory distress syndrome, which corre-

Table 1. White Blood Cell and Platelet Counts in Mice Infected with IOE

Day after infection (number of animals studied)	WBC (mean $\pm$ SD)*	Platelets (mean $\pm$ SD)*
2 (n = 3)	12,833 $\pm$ 1,352	539,000 $\pm$ 175,821
5 (n = 5)	8,041 $\pm$ 1,341	714,000 $\pm$ 261,274
7 (n = 5)	4,111 $\pm$ 3,236	205,667 $\pm$ 136,567
9 (n = 5)	2,526 $\pm$ 750	2,000 $\pm$ 2,738
Controls (n = 5)	10,778 $\pm$ 2,322	578,600 $\pm$ 119,270

*WBC and platelets $\times 10^9$ cells/L.

sponds to the pathological entity, diffuse alveolar damage.^{35,40} A few autopsied cases of HME have revealed severe diffuse alveolar damage and prominent interstitial pneumonitis.^{35,37,39} Our mouse model shows histological abnormalities diagnostic of interstitial pneumonitis. Ehrlichial organisms were clearly demonstrated in endothelial cells of the alveolar capillaries, interstitial macrophages, and marginated monocytes in branches of the pulmonary arteries. These changes most likely represent the early stages of diffuse alveolar damage that did not develop fully because of the short duration of survival of the animals infected with a lethal dose of IOE. The previous animal models reported in the literature do not describe any lung pathology except for a brief description by Shibata and colleagues²⁶ of mice infected with IOE in which ehrlichial organisms were present in mononuclear cells lining the blood vessels in the lungs. The capability of ehrlichiae to infect and grow within endothelium should expand our concept of their tropism and its pathogenetic implications. Evidence of infection of endothelial cells has not been demonstrated in humans. However, autopsy examinations of patients with HME are very scant, and full pathological evaluation of the human disease is obviously hampered. Preliminary observations by electron microscopy of a case of HME submitted to us for diagnostic immunohistochemistry did not reveal any infection of the endothelium. However, the ultrastructural study was performed under less than ideal conditions because of lack of adequate fixation and an unknown postmortem interval. Further studies are certainly needed to evaluate infection of other cell targets in HME.

One of the most common laboratory abnormalities found in humans with HME is the elevation of hepatic enzymes.^{7,8,11} Cholestasis has also been described in a few cases.⁴¹ Based on the few cases reported in the

literature, the pathology of HME in the liver includes lymphohistiocytic infiltrates, Kupffer cell hyperplasia, focal hepatocyte death, hemophagocytosis, granulomas, and microvesicular fatty metamorphosis.⁴² The histological findings in our model closely resemble the findings in the liver in HME, except for the presence of multiple foci of necrosis. The explanation for the confluent hepatic necrosis that is observed on days 7 and 9 is not apparent. No thrombi were detected in our animal model although Winslow and colleagues¹⁸ reported thrombosis in hepatic vessels of *E. chaffeensis*-infected SCID mice with coagulative necrosis in the liver. The absence of ehrlichial organisms in the areas of hepatic necrosis, a finding also reported by Shibata and colleagues,²⁶ is noteworthy. In our observations, the foci of hepatic necrosis were randomly distributed throughout the hepatic parenchyma, whereas Shibata and colleagues²⁶ described necrotic areas around the central veins. In our animal model, ehrlichial organisms were identified ultrastructurally and by immunohistochemistry both in Kupffer cells and hepatocytes. Winslow and colleagues¹⁸ described *E. chaffeensis* in sinusoidal cells consistent with Kupffer cells, and Shibata and colleagues²⁶ described the organisms of IOE in cells "lining small blood vessels in the liver." Elevations of aspartate aminotransaminases and alanine aminotransaminases on days 7 and 9 after inoculation clearly correlate with the histological findings of hepatic necrosis at days 7 and 9. Absence of elevation of alkaline phosphatase and total bilirubin levels also correlates with the absence of cholestasis or any evidence of obstruction of the biliary system. Another interesting observation in the liver was the presence of randomly distributed apoptotic bodies, generally away from the areas of necrosis. The apoptotic cells were consistent morphologically with Kupffer cells and hepatocytes. The pathogenesis of the apoptosis is at present unknown. Occasional apoptotic macrophages observed by electron microscopy on day 9 were found to be harboring ehrlichial morulae. However, double-staining of the tissue sections by the TUNEL method and anti-*E. chaffeensis* antibodies does not support the idea of apoptosis being triggered by intracellular infection by ehrlichial organisms. It would be plausible to hypothesize that apoptosis is rather a bystander phenomenon triggered by soluble factors (eg, cytokines) released from infected cells. The elucidation of the mechanisms of apoptosis in ehrlichial infections deserves future investigation.^{43,44}

Another finding in the liver was the presence of microvesicular fatty metamorphosis observed both ultrastructurally and by routine histology. The pathogenesis of this finding is unknown, although morphologically it suggests profound hepatocyte dysfunction leading to abnormal intracellular fat metabolism.

The most prominent finding in the spleen was the expansion of the marginal zone by macrophages. Areas of the red pulp were also infiltrated by macrophages. Ultrastructural and immunohistological studies revealed ehrlichial organisms present in cells lining the splenic sinusoids. In addition, the spleen contained prominent follicular hyperplasia and germinal center formation with presence of tingible body macrophages. Prominent ap-

Table 2. Hepatic Tests of Serum from Mice Infected with IOE

Days after infection	AST*	ALT†	AP‡	TB§
Day 2	154	33	106	1.2
Day 5	139	34	114	0.9
Day 7	712	155	81	1.1
Day 9	816	72	83	0.4
Control	178	43	117	1.3

*Aspartate aminotransaminase, 1U/dl.

†Alanine aminotransaminase, 1U/dl.

‡Alkaline phosphatase, 1U/dl.

§Total bilirubin, mg/dl.

optotic bodies were observed both in the red pulp and the marginal zone. The germinal centers also revealed apoptotic bodies that were probably a normal component of the immune response and unrelated directly to the infectious process because no organisms were detected in the germinal centers either ultrastructurally or immunohistologically. Shibata and colleagues²⁶ described similar splenic pathology except for the absence of follicular hyperplasia and germinal centers in their animals. The splenic pathology of HME is also similar to our findings in these mice. However, the human spleen differs somewhat histologically from the murine spleen in which hematopoiesis is normally present and lymphoid follicles are well delineated with a very well-defined marginal zone that is absent in humans.

The bone marrow is the best described target organ in humans affected by HME.⁴⁵ Findings include hypercellularity more often than a hypocellular bone marrow and with myeloid hyperplasia in the majority of cases and granulomas in most nonfatal cases. In our animal model, involvement of the bone marrow was prominent with infection of the mononuclear cells, followed by areas of confluent necrosis, and a neutrophilic response on day 9. The cellularity remained constant throughout the course.

The absence of mature granulomas in our animal model is presumably explained by the use of a uniformly lethal dose that killed the mice at an early stage of the infection. However, we have observed granulomas in the liver and bone marrow of mice infected with sublethal doses of IOE (unpublished data).

Central nervous system manifestations are prominent in a relatively small proportion (<20%) of cases of HME and consist of stupor, seizures, confusion, signs of meningeal irritation, and coma.^{8,11,36} Histopathologically, there are prominent perivascular infiltrates in the meningeal and parenchymal vessels.^{42,46} Only one of our animals showed mild alteration in the meninges that consisted of margination of mononuclear cells in the blood vessels. No parenchymal involvement was detected.

It is also worth mentioning the presence of a strong antibody response in the infected animals when tested with *E. muris* as a surrogate antigen. *E. muris* is another member of the genus *Ehrlichia* and is closely related to *E. chaffeensis* and IOE based on 16S rRNA and GroEL gene sequence analysis. The decrease in titers on day 9 is probably a manifestation of a severely ill animal that is hours away from dying of overwhelming ehrlichial infection.

A mouse model of human granulocytotropic ehrlichiosis has been developed,⁴⁷ but genetic analysis indicates that its agent, *E. phagocytophila*, is less closely related to *E. chaffeensis* than it is to *Anaplasma marginale*. Its proposed reclassification into the genus *Anaplasma* emphasizes the importance of investigation of these distinct agents in the appropriate animal models.⁴⁸ The histological, immunohistochemical, and ultrastructural pathology in immunocompetent mice infected with ehrlichial organism closely related to *E. chaffeensis*, simulates closely the alterations produced by *E. chaffeensis* in humans, and therefore this animal model provides the opportunity to study the pathogenesis and immune responses in mono-

cytotropic ehrlichiosis, using all of the tools and advantages of C57BL/6 mice.

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