Animal Model

Alveolar Septal Deposition of Immunoglobulin and Complement Parallels Pulmonary Hemorrhage in a Guinea Pig Model of Severe Pulmonary Leptospirosis

Jarlath E. Nally,* Chavit Chantranuwat,[†]
Xiao-Yang Wu,^{*‡} Michael C. Fishbein,[§]
Martha M. Pereira,[¶] João José Pereira da Silva,^{||}
David R. Blanco,^{*‡} and Michael A. Lovett*

From the Division of Infectious Diseases,* Department of Medicine, University of California Los Angeles, Los Angeles, California; the Department of Pathology,[†] Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; the Department of Microbiology, Immunology and Molecular Genetics,[‡] the Department of Pathology and Laboratory Medicine,[§] University of California Los Angeles, Los Angeles, California; and the National Reference Center for Leptospirosis,[¶] Instituto Oswaldo Cruz, Rio de Janeiro, Brazil; and the Department of Clinical Medicine,^{||} Faculty of Medicine, Fluminense Federal University, Rio de Janeiro, Brazil

Human patients suffering from leptospirosis present with a diverse array of clinical manifestations, including the more severe and often fatal pulmonary form of the disease. The etiology of pulmonary hemorrhage is unclear. Isolates of *Leptospira* acquired from patients suffering from pulmonary hemorrhage were used to develop a guinea pig model of pulmonary hemorrhage. Gross findings post-infection confirmed extensive hemorrhage in the lungs and on peritoneal surfaces as the likely cause of death. Immunohistochemistry confirmed the presence of large numbers of leptospires in kidney, liver, intestinal tissues, and spleen, but few inflammatory cells were seen. In marked contrast, few leptospires were detected in infected hemorrhagic lung tissue. Blood chemistries and hematology did not reveal the etiology of the hemorrhage observed. There was no chemical or microscopic evidence for disseminated intravascular coagulation. To ascertain an immunopathologic role during disease, immunofluorescence was performed on infected lung tissues and confirmed the presence of IgM, IgG, IgA, and C3 along the alveolar basement membrane. This suggests that an autoimmune process may

be the etiology of fatal pulmonary hemorrhage in leptospirosis. (*Am J Pathol* 2004, 163:1115–1127)

Leptospirosis continues to be a leading zoonotic infection throughout the world.^{1,2} The purpose of this study was to address the etiology and pathogenesis of the hemorrhagic diathesis seen in leptospirosis, and in particular, in the severe pulmonary form of the disease.^{3–7} The hemorrhagic potential of leptospirosis has been noted since the first description of the features of severe human infection by Weil in 1886.⁸ When the causative spirochete was first cultivated in 1916 by Inada, he accordingly named it “*spirocheta icterohaemorrhagiae*.”^{9,10} Nonetheless, appreciation that pulmonary hemorrhage can be the most prominent and often fatal manifestation of severe leptospirosis in humans has emerged relatively recently.⁵ A case of pulmonary hemorrhage in humans was first reported in 1943 in Switzerland,¹¹ by 1953 it was regarded as an infrequent but potentially severe feature of leptospirosis in the United States.¹² Small numbers of leptospirosis cases marked by severe pulmonary hemorrhage were recognized in China and Singapore by 1970.¹³ However, in 1975 a comprehensive clinical review of leptospirosis did not mention pulmonary hemorrhage *per se*.⁹

Since that time there has been growing recognition of severe cases of leptospirosis in which pulmonary hemorrhage is the most prominent and often fatal feature.^{6,7,14–17} In particular, a 1995 Nicaraguan outbreak was noteworthy because the few fatal cases presented as a hemorrhagic fever, and unlike classic Weil's dis-

Supported in part by an endowment from the Piansky Family Trust (to M.C.F.). J.E.N. is funded by a Ruth L. Kirschenstein National Research Service Award (AI055235–01) from the United States Public Health Service National Institute of Allergy and Infectious Diseases.

Accepted for publication November 25, 2003.

Address reprint requests to Jarlath E. Nally, University of California Los Angeles, Department of Medicine, 37–121 CHS, Division of Infectious Diseases, 10833 Le Conte Avenue, Los Angeles, CA 90095. E-mail: jnally@mednet.ucla.edu.

ease, renal failure was not observed.⁵ Pulmonary hemorrhage was the most frequent cause of death in this outbreak. Recent reviews of human leptospirosis have highlighted the significance of pulmonary involvement.^{1,2} It is clear that the severe pulmonary form of leptospirosis (SPFL)⁶ may occur as distinct outbreaks,^{5,14} in which a high percentage of cases are marked by pulmonary hemorrhage or as sporadic cases,^{2,6,7} while in other instances, outbreaks of leptospirosis occur in which pulmonary involvement is not striking.¹⁸ It is not known what factors influence the relative virulence and spectrum of clinical manifestations seen in human leptospirosis. Although serovar Icterohaemorrhagiae is most often associated with severe disease, it is well established that infections with diverse *Leptospira* can present with similar clinical manifestations.^{1,2,19} It is unknown whether the seeming emergence of cases of severe pulmonary hemorrhagic leptospirosis reflects unique virulence properties of specific strains.

A variety of *Leptospira* strains can cause disease in rodents with features similar to the most severe human cases. In 1957, Faine²⁰ reported that guinea pigs infected with serovar Icterohaemorrhagiae exhibited hemorrhages most prominent in lung, abdominal wall, and retroperitoneal fat. Since Faine's report,²⁰ many investigators have reported similar findings with serovar Icterohaemorrhagiae infection of guinea pigs.^{21–24} Pulmonary hemorrhage has been reported in hamsters including infections with serovar Icterohaemorrhagiae,^{25,26} serovar Pomona²⁶ serogroup Canicola,²⁷ and serogroups Hardjo and Szwaizak.²⁸ In contrast, experimental infection of adult outbred mice or rats results in asymptomatic infection and the development of chronically infected carrier hosts.^{29–31}

It is generally believed that neither thrombocytopenia nor diminution of hepatically synthesized clotting factors seen in human leptospirosis were sufficient *per se* to account for the bleeding diathesis observed.^{1,9} There have been two reports that disseminated intravascular coagulation (DIC) was associated with the hemorrhage caused by serogroup Icterohaemorrhagiae in guinea pigs.^{21,23} These reports, however, did not demonstrate significant small vessel fibrin deposition, fibrinogen consumption, or convincing release of fibrin degradation products. DIC is not mentioned as a feature of human leptospirosis.^{1,2,9}

Recently SPFL characterized by alveolar hemorrhage and acute respiratory failure was described in four patients, three of whom died within 48 hours of presentation with respiratory symptoms; blood cultures from each of the four patients were positive.⁶ The recovery of such isolates from these patients has provided an opportunity to study the causative agent of SPFL and the pathogenic mechanisms underlying pulmonary hemorrhage.

Materials and Methods

Bacteria

Isolates of *Leptospira interrogans* serovar Copenhageni were obtained from blood cultures of patients suffering

from SPFL who were admitted to Antonio Pedro University Hospital, Rio de Janeiro, Brazil.⁶ Cultures were maintained in EMJH liquid (Becton Dickinson, Cockeysville, MD) or EMJH semi-solid media (EMJH liquid media containing 0.2% noble agar). Isolates were passaged through guinea pigs to maintain virulence as described below.

Animals

Hartley male guinea pigs (Charles River Laboratories, Kingston, NY), weighing 150 to 200 grams, 10 to 15 days of age, were housed in individual cages and fed standard guinea pig chow and water *ad libitum*. Guinea pigs were injected intraperitoneally with 10³, 10⁵, or 10⁷ of low-passage RJ15958 or RJ16441 in a final volume of 500 μ l. Infected guinea pig kidney tissue, cultured in EMJH semi-solid media in the presence of 200 μ g/ml 5-fluorouracil, and sub-cultured to liquid EMJH media not more than two times provided low-passage isolates. Negative control animals were injected with EMJH media alone. Animals were monitored daily for signs of illness including weight loss and loss of mobility, and were euthanized when they appeared moribund. All animal studies were approved by the Animal Research Committee of the University of California Los Angeles.

Light Microscopic Studies

Normal and infected guinea pig viscera were fixed in neutral-buffered 4% formaldehyde (Medical Chemical Corporation, Torrance, CA), processed routinely, embedded in paraffin, cut into 4- μ m serial sections and stained with hematoxylin and eosin (H&E). Paraffin-embedded tissue sections were stained for the presence of spirochetes using the modified Steiner silver stain as previously described.³²

Immunohistochemistry

Paraffin-embedded tissue sections were incubated at 60°C for 30 minutes. Two xylene washes were applied for 5 minutes, followed by 2 $\times$ 100% ethanol for 1 minute, 2 $\times$ 95% ethanol for 1 minute, 1 $\times$ 70% ethanol for 1 minute and incubation in 3% H₂O₂ in methanol for 15 minutes. After 2 $\times$ 5-minute washes in phosphate-buffered saline (PBS), sections were incubated in 0.2 mg/ml trypsin at 37°C for 15 minutes. Sections were again washed $\times$ 2 in PBS for 5 minutes before blocking with 10% normal goat serum (NGS) (Vector Laboratories, Burlingame, CA) for 30 minutes. Primary rabbit antibody specific for outer membrane vesicles (OMV) of RJ15958 was then added at a dilution of 1:500 in 3% NGS for 90 minutes. OMV was prepared using a modified procedure as previously described.³² In brief, *Leptospira* were re-suspended in 0.1 mol/L citrate buffer (pH 3.0) and shaken vigorously for 3 hours. Samples were then passed through a French press (Thermo Spectronic, Rochester, NY) $\times$ 4 at 12,000 psi and the supernatant was centrifuged over a continuous sucrose gradient (20

to 60%). The upper OMV band was used to immunize 3.5 to 4 kg New Zealand White rabbits. After again washing tissues, biotinylated goat anti-rabbit IgG, diluted 1:200 in 3% NGS, was added for 40 minutes. Sections were again washed $\times 2$ in PBS and incubated in avidin D-horseradish peroxidase (Vector Laboratories), diluted 1:1000 in PBS for 30 minutes. After washing, staining was visualized using 3, 3'-diaminobenzidine (Vector Laboratories) for 10 minutes and the reaction was stopped using H_2O . Before mounting, slides were washed in H_2O , dehydrated by dipping in 95% ethanol 40 times followed by 100% ethanol for 40 times and 3×5 -minute washes in xylene. Sections were counterstained in weak hematoxylin.

Immunofluorescence

Fresh lung tissue from infected guinea pigs obtained at autopsy was embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and snap-frozen on dry ice. Four- μm thick frozen sections were serially cut and fixed in cold acetone for 10 minutes. Sections were air dried, washed in PBS for 5 minutes and 0.3% hydrogen peroxide in dH_2O for 10 minutes. After 3×5 -minute washes in PBS, tissue sections were blocked with 10% normal serum of the same species as the following secondary antibodies for 30 minutes. Sections were incubated in rabbit anti-guinea pig IgG, 1:50, (Sigma, St. Louis, MO), IgM μ -chain and IgA α -chain (ICN Biomedicals, Inc., Aurora, Ohio), or goat anti-guinea pig C3 (Accurate Chemical & Scientific Corporation, Westbury, NY) at 1:50 for 90 minutes and then washed in PBS for 5 minutes $\times 3$. Biotinylated goat anti-rabbit IgG or horse anti-goat IgG was then applied for 40 minutes at 1:200. After washing in PBS for 5 minutes $\times 3$, tissue sections were incubated with 1:100 FITC-Avidin D (Vector Laboratories) for 30 minutes in the dark. After final washing with PBS for 5 minutes $\times 3$, sections were mounted with Vectashield mounting medium (Vector Laboratories) and examined by fluorescence microscopy. Negative controls included negative tissue samples processed as described, and positive tissues processed without primary antibody.

Electron Microscopy

Tissues were minced into 1- to 2-mm pieces and immersed for more than 2 hours in 2% glutaraldehyde in PBS (pH 7.0). Tissue was then washed in PBS (20 minutes $\times 3$) and post-fixed in 1% osmium tetroxide. Tissues were washed again and dehydrated in graded ethanols, and embedded in Epon. One- μm sections were cut and mounted and stained with toluidine blue. From these sections, regions of interest were selected for ultrastructural study. Seventy-nm sections were cut and stained with uranyl acetate and lead citrate and examined with a JEOL JEM-100CX electron microscope.

Hematology

Blood was collected from moribund guinea pigs under anesthesia by cardiac puncture. Hematological param-

Table 1. Time of Euthanasia of Guinea Pigs after Infection with Different Amounts of SPFL Isolates RJ15958 and RJ16441

Isolate	Number of days post-infection until euthanasia	
	RJ15958	RJ16441
Dose		
10^3	10	6-7
10^5	7	5-6
10^7	6	4-5

Isolate RJ16441 was consistently more virulent than RJ15958 at similar doses resulting in all RJ16441 infected guinea pigs being euthanized sooner than RJ15958 infected guinea pigs, $p < 0.0001$.

ters were determined by IDEXX Laboratories (Sacramento, CA).

Statistics

Data from seven separate experiments were pooled for analysis using the non-parametric Wilcoxon test.

Results

Virulence of SPFL Isolates

L. interrogans serovar Copenhageni SPFL isolates RJ15958 and RJ16441 proved highly virulent when injected intraperitoneally into guinea pigs at doses as low as 10^3 leptospires (Table 1). Isolate RJ16441 was consistently more virulent than RJ15958 at similar doses resulting in all RJ16441 infected guinea pigs being euthanized sooner than RJ15958 infected guinea pigs, $P < 0.0001$ (seven separate experiments, $n = 50$).

Gross Findings

Gross autopsy revealed distinct, well-circumscribed, multi-focal areas of hemorrhage visible on surfaces of the lungs, and extensive hemorrhage on peritoneal surfaces after infection with doses as low as 10^3 leptospires (Figure 1). The hemorrhages involved the intestinal mesenteric surfaces, but not the kidneys, liver, or spleen.

Microscopic Findings

Liver

H&E staining of infected liver tissue demonstrated hepatocyte necrosis observed as groups or as scattered individual hypereosinophilic cells with pyknotic nuclei (Figure 2). Cellular discohesion of hepatocytes ranged from focal to diffuse. Increased mitotic activity and increased binucleated hepatocytes, representing hepatocyte regeneration, were observed in all animals, but were more evident in RJ15958-infected guinea pigs. Activated Kupffer cells characterized by an expanded foamy cytoplasm were commonly seen in the sinusoids. Mild to moderate increases in numbers of monocytes and neu-

Figure 1. Lungs of normal guinea pig (A) have no hemorrhage compared to those of a guinea pig 10 days after I.P. injection with 10^3 of SPFL *Leptospira* isolate RJ16441 (B). Similarly, peritoneal surfaces of normal guinea pig (C) have no hemorrhage compared to infected guinea pigs (D).

trophils were observed in portal tracts. Silver and immunohistochemical staining confirmed large numbers of *Leptospira* in infected livers (Figure 2). More spirochetes and less background staining were observed with immunohistochemical staining compared to silver stains. Thus, immunohistochemical staining with rabbit antiserum specific for outer membrane vesicles (OMV) of RJ15958 provided a higher degree of sensitivity and specificity for the detection of *Leptospira* compared to silver staining. Immunohistochemistry provided two patterns of reactivity; pattern 1 in which the staining pattern was similar in

shape to whole intact *Leptospira*, and pattern 2 in which the staining appeared as granular debris, presumably remnants of OMV or degenerated *Leptospira*. *Leptospira* were observed primarily along cell membranes of hepatocytes. Livers of guinea pigs infected with RJ16441 had larger numbers of intact leptospires identified by immunohistochemistry compared to those infected with RJ15958. However, large amounts of reactive debris were seen within the cytoplasm of activated Kupffer cells in the livers of guinea pigs infected with RJ15958. In the liver, and in all other organs, there was no evidence of

Figure 2. **a** and **b**: Liver from normal guinea pig. Note normal liver cords and sinusoidal spaces. H&E, magnification, $\times 40$ (**a**) and $\times 200$ (**b**). **c–i**: Liver from infected guinea pig. **c** and **d**: Distortion of architecture with loss of distinct cords, discohesion of cells, and necrosis of hepatocytes (N). **e**: Increase in foamy macrophages (arrow) in sinusoids. H&E, magnification, $\times 40$ (**c**), $\times 100$ (**d**), and $\times 200$ (**e**). **f**: Silver stain positive for numerous leptospires. Magnification, $\times 400$. **g**: Negative control slide for IH staining for leptospires. Magnification, $\times 100$. **h** and **i**: Positive IH staining demonstrating numerous spirochetes along cell membranes of hepatocytes. Magnification, $\times 200$ (**h**) and $\times 400$ (**i**).

vasculitis or intravascular fibrin thrombi that would occur in the presence of DIC.

Kidney

Infected kidneys were characterized by tubular cell necrosis with occasional apoptotic cells (Figure 3). Increased mitoses indicative of tubular regeneration were

noted. Occasionally, acute inflammatory cells were present in and around the renal tubules. There was also mild to marked infiltration by histiocytes. However, glomeruli appeared normal. Silver and immunohistochemical staining revealed large numbers of intact *Leptospira* (Figure 3). Intact *Leptospira* were observed along the tubular basement membrane, between tubular cells, within the tubular lumens, within the interstitium, and in

Figure 3. **a** and **b**: Kidney from control guinea pig. Note normal tubules and glomeruli (G) of cortex. H&E, magnification, $\times 40$ (**a**) and $\times 100$ (**b**). **c–h**: Kidney from infected guinea pig. **c** and **d**: Interstitial inflammatory infiltrates, with neutrophils (arrow) present in regions with tubular epithelial cell necrosis. H&E, magnification, $\times 40$ (**c**) and $\times 100$ (**d**). **e**: Silver-stained slide showing leptospires and other granular and amorphous-positive structures. Magnification, $\times 200$. **f**: Negative control slide for IH staining for leptospires. Magnification, $\times 100$. **g** and **h**: IH staining showing spirochetes in and around tubules. Fragmented granular forms are also present in **h**. Magnification, $\times 100$ (**g** and **h**).

some cases, a few were present within glomeruli. Fragments of spirochetes were found within histiocytes, in the interstitium, and in tubules. Degenerated spirochetes were rarely found in the glomeruli.

Intestine

Focal mucosal hemorrhage and edema were noted (Figure 4). The large intestine showed an increased number of foamy macrophages in the lamina propria and apoptotic bodies in the overlying epithelium. Immunohistochemical staining confirmed the presence of large numbers of intact *Leptospira* within the mucosa of the large and small intestines of guinea pigs infected with RJ15958 and RJ16441.

Figure 4. **a**: Small intestine from normal guinea pig. H&E, magnification, $\times 100$. **b** and **c**: Small intestine from infected guinea pig showing edema of the sub-mucosa and sloughing of the mucosa in **b**, and increased macrophages in the lamina propria (**c**). H&E, magnification, $\times 100$ (**b**) and $\times 200$ (**c**). **d** and **e**: IH staining for leptospires in a normal (**d**) and infected guinea pig (**e**). **f** and **g**: Normal spleen from guinea pig, compared to infected guinea pig (**h** and **i**) which demonstrates hyperplasia of germinal centers (**h**), and increased foamy macrophages in the red pulp (**i**). H&E, magnification, $\times 40$ (**f**), $\times 200$ (**g**), $\times 40$ (**h**), $\times 200$ (**i**). **j** and **k**: IH staining for spirochetes in a normal guinea pig (**j**) and an RJ15958 infected guinea pig (**k**) which demonstrates no intact spirochetes but abundant positive staining granular debris.

Spleen

Infected spleens showed reticuloendothelial system activation with increased hemophagocytosis ranging from mild to marked (Figure 4). Activated histiocytes with expanded pale to foamy cytoplasm were observed predominantly within red pulp. Immunohistochemical staining confirmed the presence of large numbers of intact *Leptospira* in the spleens of guinea pigs infected with RJ16441 (not shown). Few intact *Leptospira* were identified in guinea pigs infected with RJ15958, although reactive leptospiral debris was observed in the spleens of animals with either RJ15958 (Figure 4k), or RJ16441 and associated with the contents of enlarged foamy histiocytes.

Lung

All infected guinea pigs had significant microscopic hemorrhage in lungs involving an estimated 5 to 70% of total lung parenchyma. Early hemorrhage appeared randomly as small foci. As severity increased, coalesced areas of hemorrhage developed. Hemophagocytic histiocytes were seen in hemorrhagic alveolar spaces. Generally, there was a mild to moderate increase of interstitial cellularity of the alveolar septae due to increased numbers of mononuclear cells and occasional neutrophils (Figure 5, a to h). Increased numbers of neutrophils in capillaries were also observed. Intact leptospire were only rarely detected by immunohistochemistry. Reactive debris was occasionally detected associated with monocytes or polymorphonuclear cells.

Hematology and Chemistry

Guinea pigs injected with 10^3 or 10^7 of isolate RJ15958 or RJ16441 ($n = 10$ including 2 negative controls), were sacrificed when they appeared moribund. Blood was taken by cardiac puncture for analysis of coagulation parameters and blood chemistry (Figure 6). Fibrinogen levels in infected guinea pigs, though elevated compared to normal controls, were within the normal range of 178 to 422 mg/dl.³⁴ Platelet numbers in 4 of 6 infected guinea pigs were decreased but still adequate for normal function, ranging from 103 to 526 thousand/ μ l (normal range, 400 to 700 thousand/ μ l). Hemoglobin, hematocrit, and D-dimer (<250 ng/ml) values were similar to negative control values in all guinea pigs (data not shown). White blood cell counts in normal guinea pigs ranged from 2.1 to 3.4 thousand/ μ l compared to 3.9 to 6.7 thousand/ μ l in infected guinea pigs (mean, 5.28 thousand/ μ l). White and red blood cell morphology was normal. Monocytosis was apparent, ranging from 459 to 1254 per μ l compared to non-infected controls (34 to 84 per μ l). Prothrombin times were prolonged in some infected guinea pigs, ranging from 23.1 to 46 seconds compared to normal guinea pigs (25.1 to 25.5 seconds) (Figure 6). Similarly, partial thromboplastin times (PTT) ranged from 15.8 to 46.5 seconds in infected guinea pigs compared to normal guinea pigs (18.9 to 23.2 seconds). Collectively, these coagulation parameters suggest that pulmonary hemorrhage in guinea pigs is not due to interference of the coagulation pathway and disseminated intravascular coagulation is unlikely, as supported by negative D-dimer assays (<250 ng/ml) in all infected guinea pigs.

Blood urea nitrogen (BUN) levels (normal range, 9 to 31.5 mg/dl) were elevated in all infected guinea pigs, ranging from 44 to 129 mg/dl. However, creatine levels were within normal physiological values (0.6 to 2.2 mg/dl) for all guinea pigs,³⁵ indicating pre-renal azotemia. All infected guinea pigs had elevated bilirubin (normal range, 0.3 to 0.9 mg/dl) ranging from 2.6 to 9.1 mg/dl. Alkaline phosphatase levels in infected guinea pigs were decreased, ranging from 84 to 228 IU/L, compared to negative controls, ranging from 238 to 447 IU/L. Levels of alanine aminotransferase were also decreased in infected guinea pigs, ranging from 2 to 13 IU/L compared

to negative controls, ranging from 19 to 20 IU/L. Total protein, albumin, and globulin of infected guinea pigs were similar to negative controls.

Detection of Immunoglobulins and Complement in the Lung by Immunofluorescence

Fresh-frozen lung tissue from 22 infected guinea pigs and 4 uninfected controls was examined for the presence of IgM, IgG, and IgA and the complement component C3 by immunofluorescence (IF). Three staining patterns were distinguished in the lungs of infected animals for each immunoglobulin and C3 as shown in Figure 5 and as described in Table 2. These patterns were: staining along the alveolar septum (AS); in addition to linear staining along the alveolar septum, there was more amorphous, faint intra-alveolar staining adjacent to the alveolar surface, indicative of periseptal leak (AS + L); and intra-alveolar amorphous material filling alveolar spaces (A). The intra-alveolar pattern (A) was noted only in two animals, and probably reflects advanced destruction of alveolar structure. Table 2 indicates that of 22 infected lung tissues, 17 were positive for IgM, 15 for IgG, 13 for IgA, and 10 for C3. Each of these positives were in the AS or AS + L pattern. Of the 10 C3-positive tissues, all 10 were also positive for at least two Ig classes, and 8 of the 10 were positive for each of the three Ig classes. Distribution of IF staining for C3 in the lung sections ranged from focal in six infected guinea pigs (limited to small areas involving less than 10% of the tissue), patchy in two cases (multi-focal involving approximately 10 to 50% of the tissue), and diffuse in two cases (confluence involving approximately more than 50% of the tissue). Negative lung tissue displayed staining of lymphocytes in occasional bronchial-associated-lymphoid-tissue (BALT) and scattered discrete cells in lung parenchyma.

Electron Microscopy

Ultrastructural studies of lung tissue confirmed light microscopic studies documenting the alveolar septal wall thickening and the paucity of *Leptospira* within the lung (Figure 7). Immune complex deposits were not observed. Varying degrees of endothelial and epithelial injury, characterized by swollen, vacuolated cytoplasm were present in regions devoid of hemorrhage. Hemorrhagic regions showed more diffuse and extensive destruction of the cellular elements within the alveolar septae.

Discussion

Most human fatalities in leptospirosis are due to hemorrhage, but the etiology of hemorrhage has not been established. Pulmonary hemorrhage in leptospirosis was first reported more than a half century ago,^{11,12} but as recently as 1975, it was not mentioned as a feature of leptospirosis in a comprehensive clinical review.⁹ More recently pulmonary hemorrhage has emerged as a potentially prominent feature of the infection.^{1,3,5-7,14-17,36}

Figure 6. Fibrinogen, platelets, prothrombin time, and partial thromboplastin time (PTT). **Open diamond**, normal control, injected with media alone ($n = 2$), **square**, group 1, 1×10^7 16441 ($n = 2$), **triangle**, group 2, 1×10^3 16441 ($n = 2$), **circle**, group 3, 1×10^7 15958 ($n = 2$), and **filled diamond**, group 4, 1×10^3 15958 ($n = 2$).

In this study we used blood isolates from fatal human cases of the severe pulmonary form of leptospirosis to address the basis of hemorrhage in a guinea pig model.

The SPFL human isolates RJ16441 and RJ15958 of *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni were highly virulent for guinea pigs. 10^3 spirochetes rapidly caused fatal infection in guinea pigs weighing 150 to 200 grams, and also in guinea pigs weighing more than 500 grams (data not shown). In contrast, Adler and colleagues³⁷ have reported that there was an age-related resistance of guinea pigs to infection with serovar Pomona; animals weighing more than 100 grams were resistant to infection. In experiments conducted over the course of a year, RJ16441 proved to be reproducibly more virulent than the closely related RJ15958 passaged to the same extent *in vitro*, in that guinea pigs became moribund more quickly on infection with RJ16441. Findings at autopsy consisted of striking pulmonary hemorrhage and retroperitoneal hemorrhage without frank renal hemorrhage, just as recorded in the classic work of Faine²⁰ with serovar Icterohaemorrhagiae.

Blood chemistries and hematology did not reveal the etiology of the hemorrhage observed. Decreases in platelet numbers, observed in some guinea pigs, were not sufficient to cause spontaneous bleeding. Elevations in PT and PTT were found in some animals. There was no

chemical or microscopic evidence for disseminated intravascular coagulation, in that the D-dimer was not elevated, intravascular fibrin was not found, and fibrinogen levels were elevated, not diminished. There was also no microscopic evidence of vasculitis. Two previous studies with guinea pigs have reported DIC during infection. Higgins and Cousineau,^{21,38,39} working with serovar Icterohaemorrhagiae, described thrombocytopenia, "deposits resembling fibrin" in medium-sized arteries in the lung, and an elevation of fibrinogen degradation products in the setting of elevated fibrinogen levels, and concluded that DIC explained these findings. Pereira da Silva²³ and colleagues, working with serovar Copenhageni, also described thrombocytopenia, normal fibrinogen levels, and "slight deposits of fibrin" in capillaries of liver, kidney, and muscle, and concluded that DIC was responsible for the hemorrhagic phenomena observed, although fibrinogen degradation products were not measured. DIC is not believed to be a factor in human leptospirosis.^{40–43}

In comparing disparate results of animal studies conducted over many years it should be recognized that different *Leptospira* isolates have been used by each investigator. Additional variables to consider also include the extent of *in vitro* passage of the *Leptospira*, and the infectious dose used. Despite our finding that profound thrombocytopenia does not result from infection of guinea pigs with the SPFL isolates RJ15958 and RJ16440, the reports of Cousineau and Pereira da Silva^{21,23} indicate that there are strains of *Leptospira* serovar Icterohaemorrhagiae that do result in thrombocytopenia in guinea pigs. Regardless, our findings indicate that severe pulmonary hemorrhage due to infection with the two SPFL isolates occurs in the guinea pig in the absence of profound thrombocytopenia or DIC.

While microscopic examination of the kidneys revealed interstitial nephritis, a modest degree of tubular necrosis in the presence of leptospires in the interstitium and tubular lumens, these changes apparently were not sufficient *per se* to have had a major deleterious effect on renal function, as the BUN and creatinine of all infected animals were indicative of azotemia on a pre-renal basis, no doubt reflecting the inability of severely ill animals to drink. Electrolytes were not measured in this study, so it is not known whether electrolyte imbalance contributed to the clinical decline observed. Gross anatomical findings at autopsy implicate pulmonary hemorrhage as the cause of death due to infection with the SPFL-derived strains.

Immunohistochemistry using antiserum to outer membrane vesicles of strain RJ15958 provided visualization of intact leptospires in the gastrointestinal tract, liver, and kidneys that was superior to silver staining. The striking localization of spirochetes in intercellular spaces in the liver has

Figure 5. **a** and **b**: Lung from normal guinea pig. Note generally thin alveolar septae. **c–e**: Lung from infected guinea pig showing regions of marked hemorrhage (**c**), and thickening of alveolar septae by infiltrates of acute and chronic inflammatory cells (**d** and **e**). H&E, magnification, $\times 100$ (**a**), $\times 200$ (**b**), $\times 100$ (**c**), $\times 100$ (**d**), $\times 200$ (**e**). **f**: Normal control lung for IH staining for leptospires. Magnification, $\times 100$. **g** and **h**: IH staining of infected lung showing relatively rare leptospires present in the alveolar septae, with some granular debris present in macrophages in the alveolar septae (**h**). Magnification, $\times 200$ (**g**), $\times 100$ (**h**). **i** through **l**: Immunofluorescent staining of lung tissue. **i**: Normal control lung tissue. **j–l**: IF of lung from infected guinea pig showing prominent staining of the alveolar basement membrane (AS pattern) (**j**), prominent staining of the alveolar basement membrane with relatively faint intra-alveolar staining (AS + L pattern) (**k**) and intra-alveolar staining patterns (A). Magnification, $\times 100$ (**i**, **j**, **k**, and **l**).

been noted in previous studies,²⁴ as has the dis-cohesion of hepatocytes.^{21,38} Antigenic debris of the spirochete was readily detected in granular form within the cytoplasm of activated Kuppfer cells in the livers of guinea pigs infected with RJ15958. The spleen contained both intact spirochetes and antigenic debris within foamy histiocytes.

Lung tissue from guinea pigs infected with the SPFL strains had distinct, well-circumscribed, multi-focal areas of hemorrhage regardless of the infectious dose. The percentage of each lung tissue sample involved with microscopic hemorrhage varied considerably, ranging from an estimated 5 to 70%. However, it should be noted that lung tissue samples taken at autopsy from each guinea pig were divided for processing for H&E, immunohistochemistry, and immunofluorescence; thus the extent of hemorrhagic change found in all portions of the lungs of individual animals could not be determined.

In contrast to the abundance of intact spirochetes or their antigenic debris in kidney, liver, spleen, and large intestine, the rarity of intact leptospire as seen by immunohistochemistry in the lung was striking. The antigenic debris of leptospire was more frequently detected associated with monocytes or polymorphonuclear cells, or was in the alveolar septae. Antigenic debris was less common in the lung than in liver, spleen, kidney, or intestine, yet the lung was the tissue most damaged (in the form of hemorrhage) as a consequence of infection. Despite the paucity of leptospire and their antigenic debris in the lung, there was histological evidence of a wide-spread, yet subtle, inflammatory process. The alveolar

septae were thickened due to infiltration of monocytes and polymorphonuclear cells. Given the blebbing of endothelial cells observed by electron microscopy, endothelial injury may have engendered this inflammatory infiltrate.

The clinical phases of human leptospirosis have been termed the septic phase, referring to the first week of illness when spirochetemia can be documented by positive blood cultures, and the immune phase of the illness, which begins with the second week, and is marked by positive antibody titers to leptospiral lipopolysaccharide and leptospiruria.² Major organ dysfunction, such as cholestasis and renal insufficiency occur in the immune phase of the illness. Later still in the course of both human and equine leptospirosis, uveitis believed to be immunologically mediated may appear.^{2,44-47} In guinea pigs, antibodies to leptospiral LPS have been detected as early as 3 days after infection.³⁷

To ascertain whether immunopathology might underlie pulmonary hemorrhage, lung tissues were probed for the presence of IgM, IgG, IgA, and C3 using immunofluorescence. Three patterns of reactivity were observed in infected animals, but not in uninfected animals. The first pattern was fluorescence along the alveolar basement membrane (AS), the second pattern included fluorescence along the AS along with much fainter staining of the alveolar space. This pattern was termed AS + leak (AS + L). In the third pattern the AS was not visualized, and there was fluorescence of alveolar contents; this pattern was termed alveolar (A). The AS and AS + L

Table 2. Distribution of Immunofluorescent Patterns in Lung Tissues of Guinea Pigs Infected with 10³, 10⁵, or 10⁷ of RJ15958 or RJ16441

Guinea pig no.	Strain, dose	Day of sacrifice	IF IgM	IF IgG	IF IgA	IF C3
380	Uninfected	7	—	—	—	—
381	Uninfected	10	—	—	—	—
358	Uninfected	11	—	—	—	—
362	Uninfected	11	—	—	—	—
333	RJ15958, 10 ³	10	AS + L	AS	AS + L	—
373	RJ15958, 10 ³	10	AS	—	—	A
374	RJ15958, 10 ³	10	AS + L	AS + L	—	—
335	RJ15958, 10 ⁵	7	AS + L	AS + L	AS + L	AS + L
370	RJ15958, 10 ⁵	7	AS	AS	AS	—
391	RJ15958, 10 ⁵	7	AS	AS	AS	—
337	RJ15958, 10 ⁷	6	AS	AS	AS	AS
356	RJ15958, 10 ⁷	7	AS	AS	AS	AS
357	RJ15958, 10 ⁷	7	AS	AS	AS	—
361	RJ15958, 10 ⁷	7	AS	AS	AS	AS
375	RJ15958, 10 ⁷	7	—	—	—	—
376	RJ15958, 10 ⁷	6	AS + L	AS	—	AS + L
346	RJ16441, 10 ³	7	—	—	—	—
347	RJ16441, 10 ³	6	A	A	A	A
377	RJ16441, 10 ³	7	AS	AS	AS	AS
378	RJ16441, 10 ³	7	AS	—	—	—
348	RJ16441, 10 ⁵	6	AS	AS	AS	AS
349	RJ16441, 10 ⁵	5	—	AS	AS	—
372	RJ16441, 10 ⁵	7	AS	AS	AS	AS
393	RJ16441, 10 ⁵	6	AS + L	—	AS + L	—
371	RJ16441, 10 ⁷	4	AS + L	AS + L	—	AS
379	RJ16441, 10 ⁷	4	—	—	—	—

Three staining patterns were observed for IgM, IgG, IgA, and C3 as shown in Figure 5 including: staining along the alveolar septum (AS); in addition to linear staining along the alveolar septum, there was more amorphous, faint intra-alveolar staining adjacent to the alveolar surface, indicative of periseptal leak (AS + L); and intra-alveolar amorphous material filling alveolar spaces (A).

Figure 7. Electron photomicrographs of lung from infected animals. **a:** Marked widening of an alveolar septum. There is congestion of capillaries that are filled with erythrocytes (E). Neutrophils (N) and macrophages (M) are also present. **b:** Congested capillaries distended by erythrocytes (E) and damaged endothelial cells with cytoplasmic blebs (B). No intact leptospires or dense deposits were observed. Uranyl acetate/lead citrate stain, magnification, $\times 3328$ (**a**), $\times 10352$ (**b**).

patterns were indicative of specific binding of immunoglobulins and complement to the AS. The alveolar pattern (A) was seen in only 2 of the 22 infected guinea pigs with pulmonary hemorrhage. In one, there was also deposition of IgM along the alveolar septum, but intra-alveolar C3 was seen. In the second animal, only intra-alveolar IgM, IgG, IgA, and C3 were detected. The fact that pattern A was so infrequently seen in lung tissues despite the prominence of intra-alveolar hemorrhage probably indicates that pattern A was not merely the consequence of intra-alveolar blood. Of the remaining infected guinea pigs, 17 were positive for IgM, 15 for IgG, 13 for IgA, and 10 for C3 in a striking pattern confined to the AS, or involving the AS plus a component of leak into alveoli. In every case in which there was C3 deposition along the

AS, there was also deposition of IgM and either IgG or IgA. This finding suggests that complement activation had occurred due to immunoglobulin binding. Overall, the AS was detected by immunofluorescence in 20 of the 22 guinea pigs, but in none of the four uninfected control animals.

The immunofluorescence patterns observed could have resulted from the following sequence of events: binding of IgM, IgG, and/or IgA to target(s) in the alveolar septum; complement activation, leading to the deposition of C3; resultant damage to the alveolar septum; and leakage of cells, as well as hemorrhage into alveoli. This scenario would explain the putative periseptal leak pattern and the intra-alveolar patterns of staining observed. The reasons for failure to detect C3 in 12 of the 22 infected lung tissues are unclear but may include the short half-life of C3⁴⁸ or sampling error since the extent of injury was quite variable from region to region within the lung. In any case, it was clear that the areas having AS and AS + L patterns of deposition of antibodies and C3 far exceeded the areas in which leptospiral antigens were detected by immunohistochemistry. This suggests that the bound immunoglobulins observed were directed at an autoantigen. Because immunohistochemistry was conducted using antiserum raised against the outer membrane of leptospires cultivated *in vitro*, it is tempting to hypothesize that a novel leptospiral antigen is expressed only during infection, and is distributed far more widely within the alveolar septum than other leptospiral antigens. An alternative explanation could be the up-regulation of complement and/or immunoglobulin receptors in response to infection.

The anatomy of the alveolar septum is complex.⁴⁹ By immunofluorescence, deposition of immunoglobulin may be granular (classical immune deposits as occur in certain renal diseases), or linear (as occurs in other renal diseases and Goodpasture's syndrome). Granular deposits are visible by immunofluorescence, electron microscopy, and sometimes even by light microscopy. Linear deposits are seen by immunofluorescence, but usually not by electron microscopy. The pathogenesis of the lung disease in this experimental system best fits with a model of linear deposition of immunoglobulin and complement as occurs in Goodpasture's syndrome or anti-GBM disease. The inflammatory infiltrate of monocytes and PMNs observed in thickened alveolar septae included some cells in which leptospiral antigen was demonstrated by immunohistochemistry. There are several possibilities to explain the presence of inflammatory cells observed in the alveolar septum: antigenic leptospiral debris found within the alveolar septum could reflect the clearance of intact spirochetes by inflammatory cells; endothelial damage evidenced by the blebbing formation of endothelial cells seen by electron microscopy could have drawn an inflammatory response; or, finally, complement activation evidenced by the detection of C3 could have caused the inflammation. In this study, animals were sacrificed when moribund. Temporal study of the numbers of leptospires and amount of leptospiral antigen, of the numbers of inflammatory cells, and of the deposition of antibodies and complement components

as a function of the duration of infection might provide further insight into pathogenesis. Overall, despite the large burden of spirochetes in many tissues, the inflammatory response was strikingly muted. Except for very few neutrophils, predominantly in the lung, and in liver, kidney, spleen, and intestine, where there were large numbers of spirochetes, there were only macrophages, presumably ingesting degenerating spirochetes. The meager inflammatory response to an overwhelming fatal infection may indicate that *Leptospira* employs a specific mechanism to diminish the magnitude of the host immune response.

The recently published genome sequence of *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar Lai included sequences indicating the existence of three leptospiral proteins related to mammalian platelet activating factor acetylhydrolase, to type A domains of von Willebrand factor (VWF), and to paraoxonase.⁵⁰ It was suggested that the potential activities of these proteins with hemostasis related functions in combination with the activity of leptospiral collagenase could result in hemorrhage. The observations made in this study may be of relevance to this hypothesis. Large numbers of leptospire were found in guinea pig liver and kidney, without evidence of hemorrhage. Hemorrhagic lung tissue contained only rare leptospire. These findings indicate that a high concentration of leptospire in a tissue do not necessarily lead to hemorrhage in that tissue.

Endothelial cell injury and vasculitis have been cited as features of the pathology of leptospirosis^{2,9} but their documentation in the literature is limited. DeBrito and colleagues^{22,51–53} examined diaphragm, kidney, and lung of guinea pigs by light and by electron microscopy. Vascular damage consisted of focal damage to capillaries. Opening of endothelial intercellular junctions and endothelial cell swelling were noted. Large endothelial blebs were noted in pulmonary capillaries by electron microscopy. Nicodemo and colleagues⁴¹ examined lung tissue from humans who died from leptospirosis. Edema of alveolar septae and an infiltration of macrophages and lymphocytes, and endothelial cell swelling, generally mild to moderate, were the principal findings. In this study there were no signs of systemic vasculitis, and damage of pulmonary endothelium at the level of electron microscopy was limited to endothelial cell blebs. There was no evidence of loss of integrity of endothelial intercellular junctions in lung tissue.

The presence of antibody along the alveolar septal wall in the infected guinea pigs suggests an autoimmune response may be in play, perhaps similar in mechanism to Goodpasture's disease. Goodpasture's disease is characterized by development of antibodies to components of type 4 collagen of the renal tubular basement membrane, which cross-react with type 4 collagen in the lung resulting in damage to the alveolar septae and hemorrhage.⁵⁴ However, examination of renal tissue in this study did not reveal focal or segmental glomerulonecrosis characteristic of Goodpasture's disease before development of extensive destruction of the glomerular basement membrane. In fact, glomeruli appeared normal, despite the presence of a few intact leptospire within

glomeruli. Further ultrastructural analysis of alveolar septal architecture in fatal human cases, along with search for deposition of immunoglobulins and complement components along the alveolar septum are clearly needed.

Definitive demonstration that an autoimmune process is the etiology of fatal hemorrhagic complications of leptospirosis, would suggest that novel therapeutic interventions to remove autoantibodies from blood and reduce their synthesis need to be explored.

Acknowledgments

We thank Longsheng Hong and Ya-Ying Wang for their excellent technical expertise. We also thank the Division of Laboratory Animal Medicine. In addition, we thank SungChing Glenn for statistical analyses and Dr. James N. Miller, Chairman of the UCLA Animal Research Committee, for helpful discussions.

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