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EXPERIMENTAL LEPTOSPIROSIS IN MARMOSET MONKEYS (*CALLITHRIX JACCHUS*): A NEW MODEL FOR STUDIES OF SEVERE PULMONARY LEPTOSPIROSIS

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Abstract. Experimental infection of marmoset monkeys (*Callithrix jacchus*) with *Leptospira interrogans* serovar Copenhageni showed microscopic patterns of tissue reactions comparable to those seen in the severe forms of human leptospirosis, including intra-alveolar hemorrhage. The most impressive microscopic changes were seen in the lung and kidney of animals killed at days 6 and 12 after inoculation. There were extensive and irregular areas of hemorrhage predominating around main bronchial branches or diffusely spread to the pulmonary parenchyma, as well as severe tubulointerstitial nephritis. Antibody response detected by the microscopic agglutination test was quantitatively similar to those seen in humans and paralleled severity of tissue lesions. The distribution of leptospires or antigenic debris in infected tissues was observed by immunofluorescence and confocal laser scanning microscopy. Large numbers of typical leptospires were seen in the lumen of proximal renal tubules. Positive reactions showing antigenic debris were closely associated with sites of tissue damage.

INTRODUCTION

Leptospirosis is a worldwide bacterial zoonosis caused by several species of invasive spirochetes belonging to the genus *Leptospira*.[1] Severe forms of human leptospirosis continue to pose challenges to clinical practice.[2–4] Several publications have drawn attention to the severe pulmonary form of leptospirosis (SPFL) in Brazil.[5,6] Pulmonary findings for patients with leptospirosis are relatively common, but these manifestations are usually mild and often overshadowed by manifestations of other organ-system involvement.[7,8] The severe pulmonary form of leptospirosis is the most severe clinical presentation of the disease and has a rapid clinical course with high mortality rates. Deaths may occur in less than 72 hours after the advent of respiratory signs and symptoms, which usually appear between the fourth and the sixth day of disease.[9] In retrospect, severe pulmonary hemorrhage due to leptospirosis has been recognized worldwide. For example, epidemic leptospirosis associated with pulmonary hemorrhage has been reported in Nicaragua[10] and in China.[11] Epidemic outbreaks or isolated cases in which pulmonary hemorrhage appears as a dominant and life-threatening clinical feature have been recently reported in Brazil, Nicaragua, Australia, Argentina, Korea, Thailand, India, and Seychelles.[9,10,12–16]

It is not known what factors influence the virulence and spectrum of clinical manifestations seen in human leptospirosis. *Leptospira interrrogans* serovar Copenhageni has been isolated from case with SPFL, as well as from cases showing the classic signs and symptoms of Weil's disease in Brazil.[9,17] However, similar serovars have been isolated in the absence of severe disease.[18] *Leptospira interrogans* serovar Valbuzzi has been reported to cause severe pulmonary hemorrhage in the Andaman Islands.[15] *Leptospira interrogans* serovar Lai has been isolated from SPFL cases in China, leading to studies on genomic sequencing of *Leptospira*.[11,19,20] It should be stressed that there are a very small number of SPFL clinical isolates available from the field.

The rationale of this study was to establish an experimental model for studies on the pathogenesis of severe leptospirosis, with a particular focus on pulmonary hemorrhage. In this report, we describe tissue changes in the lung, liver, kidney and skeletal muscle seen in the experimental infection of marmoset monkeys, as well as the expression and distribution of leptospiral antigens in tissues.

MATERIALS AND METHODS

Non-human primates. Eleven (six females and five males), young, adult, wild-caught marmosets (*Callithrix jacchus*) were kindly donated by the Sao Carlos Zoo (Sao Paulo, Brazil). They had body weights ranging from 240 to 460 grams (mean ± SD body weight = 313.3 ± 57.5 grams in females and 318 ± 34.9 grams in males). All animals were serologically negative for infection with *Leptospira* by microscopic agglutination test (MAT) using the following serovars: Andamana, Australis, Autumnalis, Bataviae, Canicola, Castellonis, Copenhageni, Celledoni, Shermani, Cuica, Cynopteri, Djasiman, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Patoc, Pomona, Pyrogenes, Saxkoebing, Sejroe, Sentot, Tarassovi, and Wolffi. Marmosets were kept at a constant temperature (25 ± 1°C), a relative humidity of 60 ± 5%, and 12–hour intervals of light and darkness. The animals were caged individually and feed with marmoset diet supplemented with fruits and water *ad libitum*. Institutional approval for studies on experimental leptospirosis using marmoset monkeys were obtained from Ethical Committee of the Oswaldo Cruz Foundation/FIOCRUZ and The Brazilian Institute for Environment and Renewable Resources. Maintenance and care of experimental animals complied with procedures outlined by the Interagency Primate Steering Committee.[21]

Experimental infection. The master culture was originally isolated from a fatal human case with the severe pulmonary form of leptospirosis.[9] The isolate is available for research purposes at the culture collection of The National Reference Center for Leptospirosis, Oswaldo Cruz Institute (Rio de Janeiro, Brazil). It was identified as serovar Copenhageni with monoclonal antibodies (MAbs) F70C14, F70C24, and F89C12 (Royal Tropical Institute, Amsterdam, The Netherlands).

TABLE 1
Serum chemical values in *Callithrix jacchus* infected with *Leptospira interrogans* serovar Copenhageni*

Monkey	pid	BUN, mg/dL	Creatinine, mg/dL	AST, IU/L	ALT, IU/L	BT, mg/dL	BI, mg/dL	BD, mg/dL
Cj-01	1	71	0.4	204	33	0.74	0.37	0.37
Cj-02	3	44	0.5	204	34	1.11	0.37	0.74
Cj-03	6	195	1.9	470	47	5.92	5.18	0.74
Cj-04	9	146	1.0	218	37	8.88	6.29	2.59
Cj-05	12	104	1.7	218	44	19.24	12.95	7.29
Cj-06	15	57	0.6	204	39	1.11	0.37	0.74
Cj-07	18	65	0.5	110	30	0.74	0.37	0.37
Cj-08	21	57	0.5	198	30	0.74	0.37	0.37
Cj-09	24	47	0.9	124	36	1.11	0.37	0.74
Cj-10	28	36	0.6	114	28	0.74	0.37	0.37
Cj-11	NI	30	0.6	110	28	0.74	0.37	0.37

* pid = post-inoculation day; BUN = blood urea nitrogen; AST = aspartate aminotransferase; ALT = alanine aminotransferase; BT = total bilirubin; BI = unconjugated bilirubin; BD = conjugated bilirubin; NI = non-infected.

The first passage, derived directly from the master culture, was grown in 10 mL of Ellinghausen and McCullough medium. Leptospires were counted by dark ground microscopy using a Petroff-Hausser chamber. A total of 2.7×10^6 leptospires was inoculated into each marmoset by the intraperitoneal route. One marmoset was inoculated with sterile medium and maintained under the same conditions as the experimentally infected animals. Monkeys were killed on pid 1, 3, 6, 9, 12, 15, 18, 21, 24, and 28. Results of clinical examinations and anatomic observations were kept. Animal biosafety level 2 procedures were used.

Serum chemistry, serologic, and isolation procedures. Up to 2 mL of blood were drawn from the femoral vein for biochemical assays, serologic analysis, and blood culture. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, blood urea nitrogen, and creatinine were measured in the monkeys killed at pid 1, 3, 6, 9, 12, 15, 18, 21, 24, and 28. Blood cultures to recover leptospires and serologic tests were carried out on survivors at the above-mentioned time intervals. Living leptospires derived from the master culture (*L. interrogans* serovar Copenhageni) were used as antigens for the MAT. The MAT and attempts to recover leptospires were performed according to procedures previously described.[22] In addition to the blood cultures, samples from kidneys and urine were processed for leptospires isolation at the above-mentioned time points.

Histopathologic studies. The lung, liver, kidney, and skeletal muscle were examined for pathologic changes by light microscopy. Paraplast-embedded tissues were sectioned at 4 μm and routinely stained with hematoxylin and eosin and Giemsa.[23]

Immunofluorescence. Sections of paraplast-embedded tissues of lung, liver, kidney, and skeletal muscles were sectioned at 4 μm. Leptospires or leptospiral antigens were labeled with rabbit immune sera prepared with heat-killed leptospires derived from the master culture according to procedures previously described.[24–26] Mouse MAb F70C24 (Royal Tropical Institute) reactive with serovar Copenhageni by the MAT was used to improve the sensitivity and specificity of the immunofluorescence detection, especially in the lung. Slides were counterstained with Evans blue and observed with an LSM 410 confocal laser-scanning microscope using Ar 488 nm and He/Ne 543 nm lasers (Carl Zeiss, Oberkochen, Germany).

RESULTS

Clinical observations and gross anatomy. Infection with leptospires was characterized by weight loss and dehydration. The most significant weight loss was seen at pid 6–12 (mean ± SD = 11.8 ± 5.7% by pid 6). Gross autopsy showed well-circumscribed, multi-focal areas of hemorrhage on the pleural surfaces of the lungs and extensive hemorrhage on peritoneal surfaces in the monkeys killed on days 6–12. Jaundice was present in the subcutaneous tissue and viscera. Because monkeys were killed at previously determined time points, it is not known whether the severely ill monkeys killed on pid 6, 9, and 12 would have survived.

Serum chemistry. Values for each marmoset are shown in Table 1. Hyperbilirubinemia, predominantly conjugated, and moderately increased aminotransferase levels were detected in monkeys killed on pid 6, 9, and 12. Elevated levels of blood urea nitrogen and creatinine was also found, with highest levels on pid 6, 9, and 12.

Bacteriologic and serologic follow-up. Leptospires were recovered from blood on pid 1 until pid 9 and from kidney or urine after pid 6. Antibodies to leptospires were detected by the MAT after pid 6; peak titers were seen at pid 15 and 18, and these remained at plateau levels in the monkeys killed on pid 21, 24, and 28 (titers ranged from 1:100 to 1:204,800).

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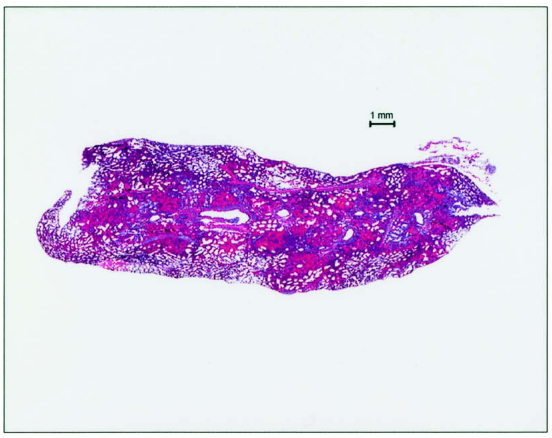
FIGURE 1. Histologic findings in marmoset monkeys inoculated with *Leptospira interrogans* serovar Copenhageni. **A**, Lung at post-inoculation day (pid) 9. Scan power view showing a section of a pulmonary lobe. **B**, Multiple areas of intense alveolar hemorrhage (original magnification × 200). **C**, Pulmonary section showing thickened septa due to an increase in interstitial cells (original magnification × 400). **D**, Liver six days after infection showing a small area of focal necrosis with adjacent cells (original magnification × 400). **E**, Kidney at pid 3 showing mild acute tubular necrosis (original magnification × 400). **F**, Kidney at pid 6 showing interstitial inflammatory infiltration mainly around the glomeruli (original magnification × 200). **G**, Kidney at pid 9 showing interstitial nephritis that is aggravated and more widespread. Many dilated tubules contain different types of casts (original magnification × 200). **H**, Interstitial hemorrhage in skeletal muscle with degenerative changes at pid 9 (original magnification × 400). (Hematoxylin and eosin stained.)



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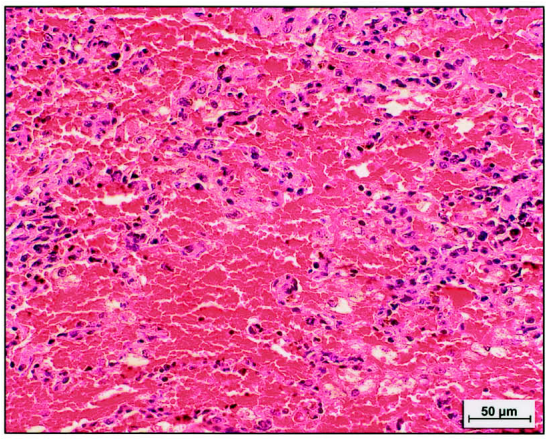

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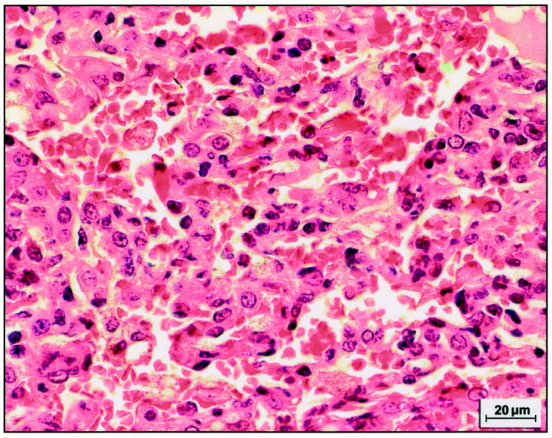

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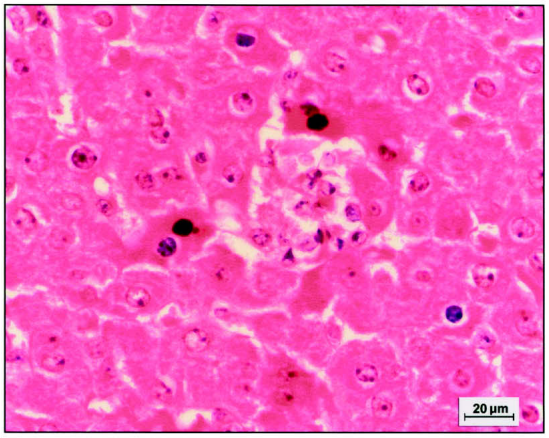

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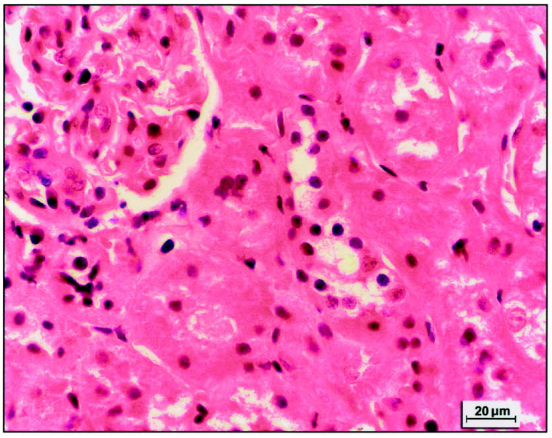

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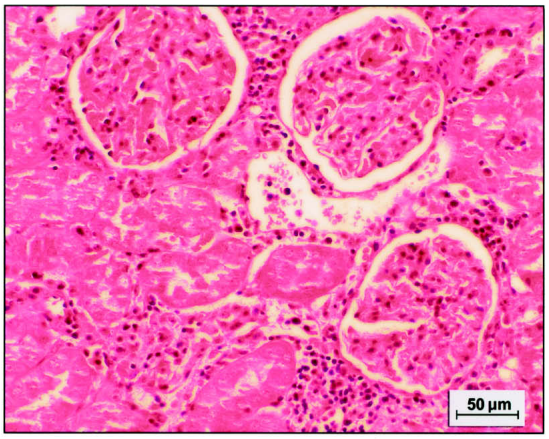

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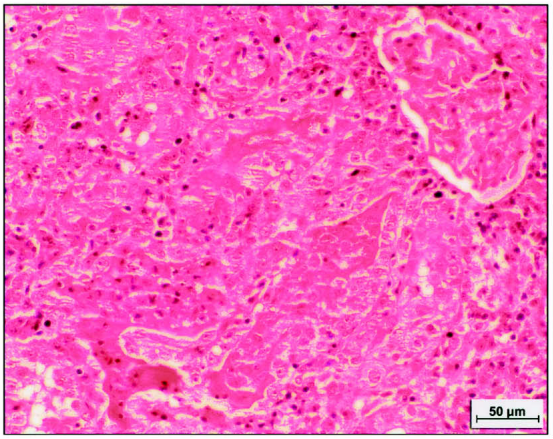

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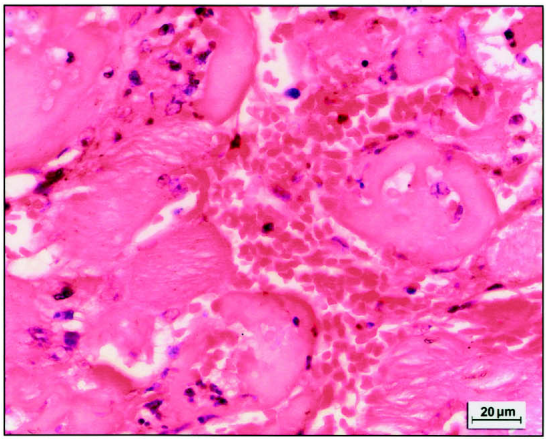

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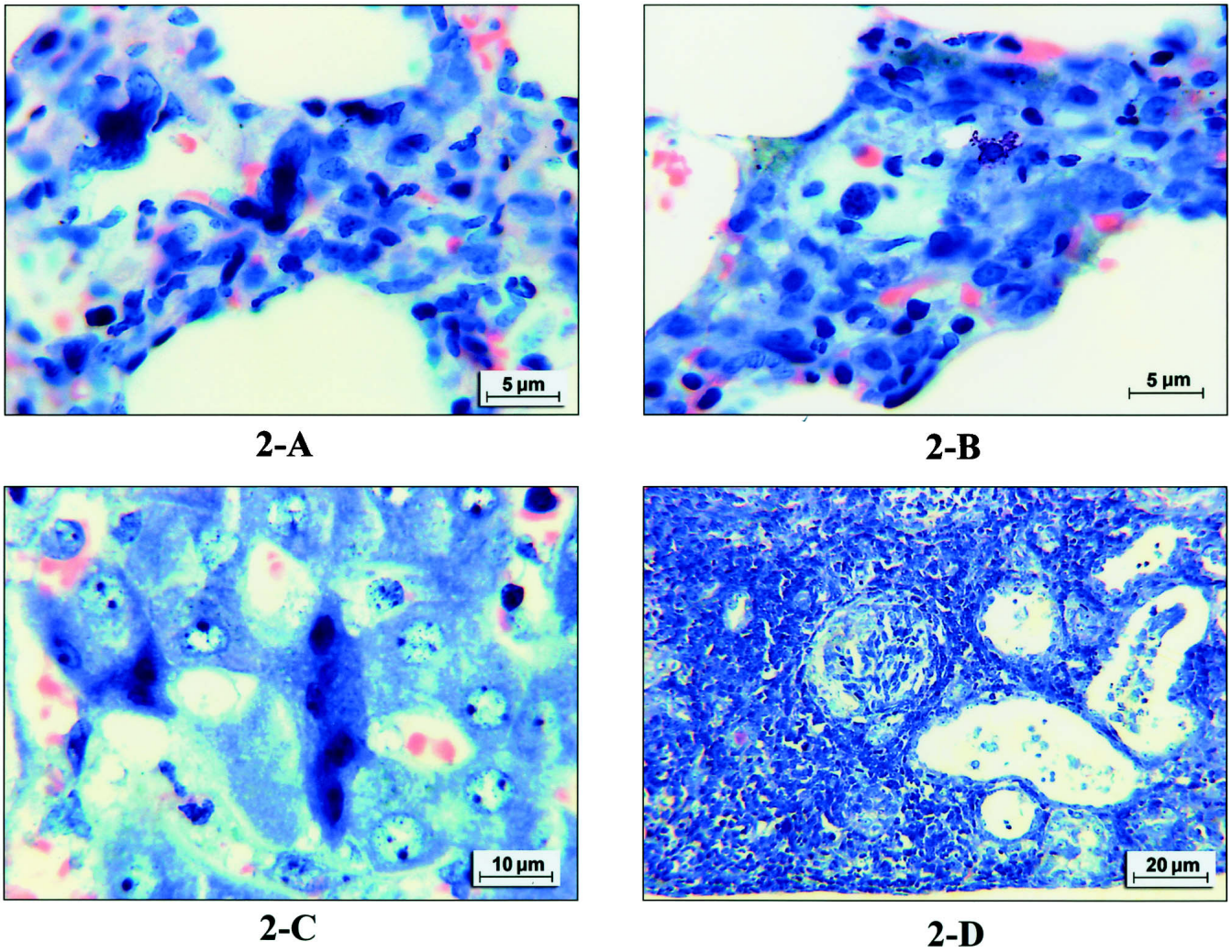

FIGURE 2. Histologic findings in marmoset monkeys inoculated with *Leptospira interrogans* serovar Copenhageni. **A**, Megakaryocytes inside vessels of thick alveolar septa, which also display large numbers of macrophages and some mature neutrophils (magnification × 500). **B**, Lung at post-inoculation day (pid) 12 showing the presence of degranulated mast cells in the alveolar septum, which is thickened by macrophages with or without hemosiderin pigment, lymphocytes, and few neutrophils (magnification × 500). **C**, Liver at pid 6 showing hyperthrophic Kupffer cells, lymphocytes in sinusoids, and condensed hepatocytes with dark and retracted nuclei tending to picnosis (magnification × 310). **D**, Kidney at pid 9 showing intense interstitial nephritis and dilated tubules with cellular cylinders and mesangial proliferation in one isolated glomerulus (magnification × 120). (Lennert's Giemsa stained.)

Morphologic findings. Several distinct microscopic patterns of tissue damage were identified in lung, liver, kidney, and skeletal muscle. The most impressive lesions were observed in monkeys killed at post-inoculation day (pid) 6, 9, and 12. The main histopathologic changes are described in the following sections.

Lung. Early after leptospiral infection, lungs were congested and alveolar spaces contained proteinaceous fluid and erythrocytes. At pid 3, intra-alveolar hemorrhagic areas first appeared. At pid 6, 9, 12, and 15, hemorrhagic phenomena were more prominent, manifested by confluent patchy areas and presence of hemosiderin-laden macrophages in alveolar spaces and in the interstitium of septa (Figure 1A and B). Alveolar septa were thickened by inflammatory infiltrates consisting of macrophages, neutrophils, lymphocytes, and occasional mast cells (Figures 1C and 2A and B). The septal vessels of infected animals were congested, containing higher number of megakaryocytes than the controls (Figure 2A). Only areas of interstitial congestion, without hemorrhage, and/or septal infiltration by inflammatory cells were detected in the animal killed at pid 28.

Liver. Inflammatory changes consisting of mild interstitial edema, vascular congestion, and focal necrosis were found in the infected marmosets. The changes began to appear from the first day of infection, gradually progressing until pid 9. There was occasional hepatocyte vacuolization, and dissociation of the liver parenchymal cells, but this was not found in all cases. Lobular architecture was generally preserved. Moderate bile stasis was detected in bile ducts. By day 6, randomly distributed foci of necrosis and degenerative changes were observed in many lobules (Figures 1D and 2C). This animal also showed sinusoidal and portal congestion and areas of hemorrhage in a few lobules. Councilman bodies were occasionally observed extruding into the sinusoids with activation of Kupffer cells, some of which containing ingested debris. Mild fatty change was also detected.

Kidney. The principal changes in the kidneys were found in the tubules and interstitial tissues. The first changes occurred in epithelial tubular cells, characterized by acute tubular necrosis without reactive inflammatory cells (Figure 1E). After day 6, tubular dilation was prominent, containing several types of proteinaceous and cellular casts. (Figures 1G and 2D). With the progression of infection, severity of the parenchymal lesions varied, showing extensive inflammatory infiltration. Interstitial nephritis was a major finding, sometimes intense, being more striking in the peri-glomerular interstitium (Figures 1F and 2D). Minor, focal changes were seen in the glomeruli (Figures 1F and G and 2D).

Skeletal muscle. From day 9 onward marmosets showed interstitial edema and leakage of erythrocytes, lysis, and regenerative muscular changes with mild focal necrosis (Figure 1H). In the monkey killed on pid 12, lesions were more prominent, showing hemorrhagic foci and multifocal necrosis. There was a mild inflammatory infiltrate by mononuclear cells.

***In situ* presence of leptospiral antigens.** Two staining patterns were distinguished by immunofluorescence using the rabbit polyclonal antisera and MAbs. The first pattern consisted of intact and well-shaped leptospires found in the liver and isolated or clustered in the renal proximal tubule lumen, as determined with rabbit polyclonal antisera (Figure 3D and E). In contrast to the distribution of leptospires and leptospiral antigens in the tubular lumen, little antigen was observed at the sites of interstitial inflammatory infiltrates (Figure 3D). Intact leptospires were only rarely detected in the lung by polyclonal rabbit antisera. A second pattern consisted of amorphous antigenic material appearing to be composed of bright granules. This was predominantly seen using the MAbs. A smear of cultivated leptospires showed that isolated or clustered leptospires had the same appearance if they were stained by the same technique using the MAbs as the primary antibody. This finding was in contrast to the use of rabbit polyclonal antisera that revealed intact leptospires. Immunofluorescence with MAbs was more sensitive than rabbit antiserum in detecting leptospiral antigens in hemorrhagic lung, as well as in liver, kidney, and skeletal muscle (Figure 3A, B, C, and F). Leptospiral antigen was seen closely associated with areas of tissue damage, particularly on pid 9, 12, and 15. In control experiments, normal rabbit serum, normal mouse serum, and phosphate-buffered saline did not show reactivity to infected tissues. Uninfected tissues did not show any positive reaction.

DISCUSSION

The highly virulent clinical isolate obtained from humans with SPFL caused similar disease in marmoset monkeys under experimental conditions. The major tissue changes were comparable to human post-mortem findings by pid 9 and 12. The clinical spectrum of leptospirosis has changed fairly recently in some geographic areas with the emergence of SPFL.[5,6,10] It is not known whether unique virulence properties of specific strains are associated with the seeming emergence of SPFL.[9,15,20]

Young adult marmosets infected with 2.7×10^6 leptospires developed severe pulmonary hemorrhage, along with hepatic injury, as shown by conjugated hyperbilirubinemia, severe tubulointerstitial nephritis, and hemorrhagic necrosis of skeletal muscles. These changes have been well described in human cases of leptospirosis and in guinea pigs.[9,27–30] There was clinical and microscopic evidence of recovery in survivors at pid 21, 24, and 28. Because monkeys were killed at previously determined time points, it was not possible to predict whether or how long severely ill monkeys would survive after infection. Further studies using lower doses, different inoculation routes, and extended observation times will provide more comprehensive data about lethality rates, kinetics of antibody responses, the duration of the renal carrier state, and practical use of the model for tests with drugs and vaccines.

When comparing a small number of different studies of experimental leptospirosis in nonhuman primates over many years, it should be recognized that different species of monkeys and different serovars of *Leptospira* produce variable results from which firm conclusions are difficult to obtain. Grivet monkeys infected with *L. borgpetersenii* serovars Hardjo, Balcanica, and Tarassovi showed no signs of severe clinical disease.[31–33] Additional variables influencing the expression of severe leptospirosis also include the extent of *in vitro* passage of the infecting strains of *Leptospira*, the infectious dose used, and route of inoculation. In this study, we used fresh isolates of highly pathogenic leptospires obtained from the blood of a human case of SPFL.[9]

Natural infection of monkeys by leptospires is an unusual event, but has been found in serologic surveys or under exceptional conditions in captivity.[34,35] *Callithrix jacchus* has not been found to be a susceptible host or natural carrier of leptospires. The only reported attempts to reproduce the disease in marmoset monkeys were by Noguchi and others in 1924 in describing an epidemic outbreak of yellow fever in Brazil.[36] This is linked to a historical error in relation to the etiologic agent of yellow fever, and no conclusive results about reproducing the disease by inoculation of the so-called *L. icteroide*s in marmosets were ever reported. The common marmoset (*C. jacchus*) appears to be a good model for studying pulmonary hemorrhage due to leptospirosis. It is a small New World primate native to northeastern Brazil with increasing numbers in reserves in the southeastern region of the country. It has been used in biomedical research since the early 1960s.[37] Use of this species for research purposes continues to grow at a rapid pace because they are a viable alternative to other nonhuman primate species. There is extensive data on the cross-reactivity of anti-human monoclonal antibodies with marmoset surface receptors on white blood cells, as well as similarities and differences in the structure of a variety of surface receptors between *C. jacchus* and humans.[38] The marmoset model could be used for further studies on the cellular and molecular mechanisms involved in SPFL. It has advantages when compared with guinea pigs and hamsters and if one considers the large amount of immunochemicals that are available for research.[29,30] Several mice strains have failed to reproduce the disease.[39,40] In a previous study, we found that young inbred C3H/He mice are also a promising tool, but it is not the ideal model since there are variations in susceptibility according to age. Mice more than three weeks old become more resistant to infection.[41]

The most impressive histopathologic finding of infected marmoset monkeys was the intra-alveolar hemorrhage with interstitial inflammatory infiltrate. It was seen as an early feature of the disease being observed from pid 3 to pid 6, 9,

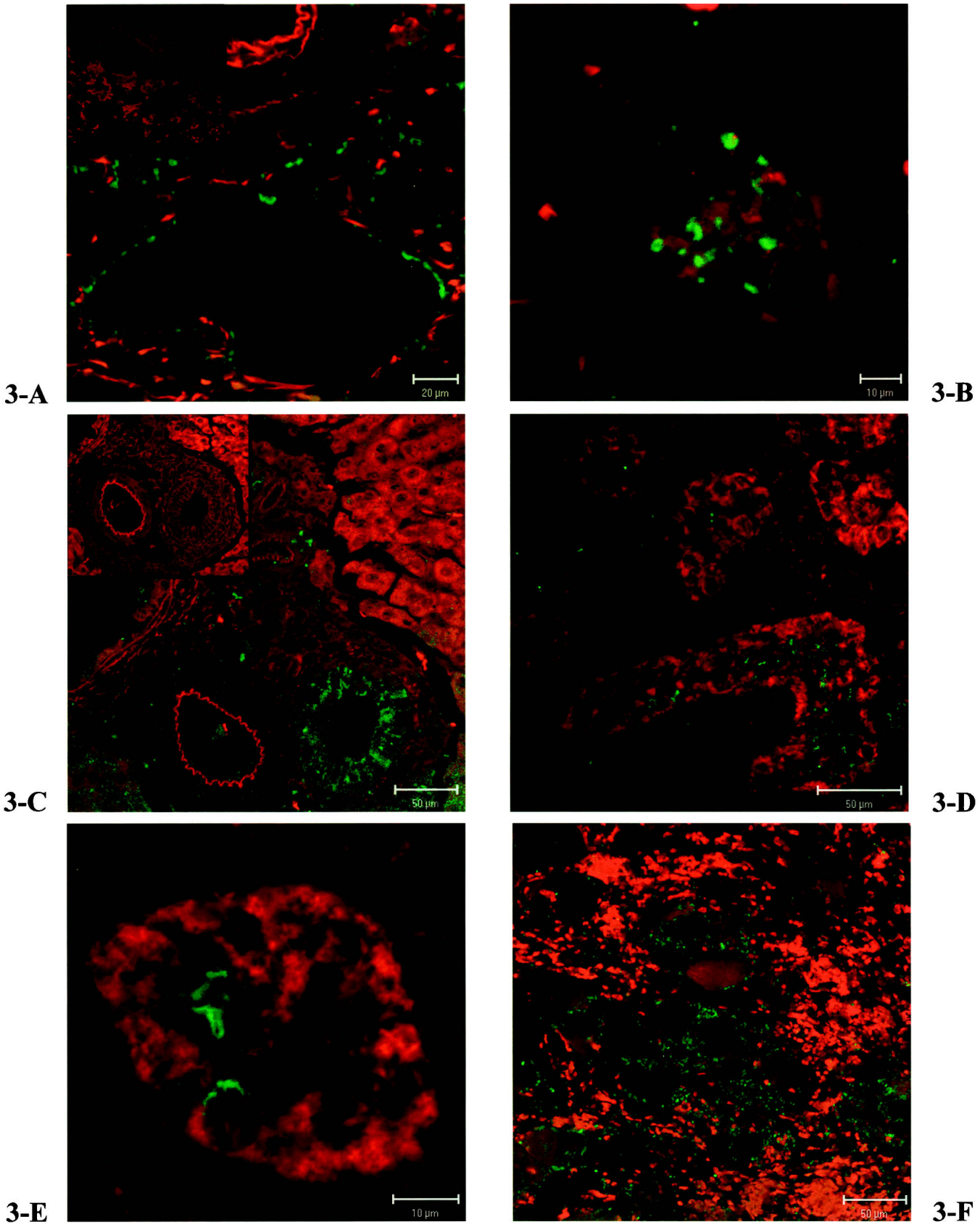

FIGURE 3. Expression and distribution of leptospiral antigens in marmoset monkeys inoculated with *Leptospira interrogans* serovar Copenhageni viewed by confocal laser scanning microscopy. **A**, Lung of a marmoset monkey killed at post-inoculation day (pid) 9 showing an telectasic area with antigens located in undefined alveolar cells (monoclonal antibody [MAb] F70C24. The **inset** shows the reaction control. **B**, Liver of a marmoset monkey killed at pid 6 showing antigen deposits forming clumps in small foci of parenchymal necrosis (MAb F70C24). **C**, Liver at pid 6 showing a positive reaction in the portal biliary ductal cells (MAb F70C24). The **inset** shows the reaction control. **D**, Kidney of a marmoset monkey killed at pid 9 showing large amounts of leptospires within the proximal tubular lumens. Rabbit immune serum was used as the primary (polyclonal) antibody. **E**, Leptospires lining the border of epithelial tubular cells. Rabbit immune serum was used as the primary (polyclonal) Antibody. **F**, Skeletal muscle at pid 9 showing leptospiral antigens deposited around skeletal muscle fibers transversally sectioned and interstitial hemorrhagic foci (MAb F70C24). (Fluorescein isothiocyanate label [**green**] counterstained with Evans blue.)

and 12 (Figure 1A and B). It is a peculiar morphologic feature that seems to be produced by an intense inflammatory response with extravasations of red blood cells out of the capillary bed. It should be stressed that this feature is not seen in other capillary-leak syndromes progressing with acute respiratory distress such as dengue hemorrhagic fever or hantavirus pulmonary syndrome. The proposed contributing factors to the bleeding disorders remain speculative. Among the diverse hypotheses to explain the hemorrhagic diathesis, two are of particular importance since the widespread injury to the endothelial cells is thought to be a major event. One is that direct injury to the small blood vessels is caused by a putative toxin. A second is that there may be immune-mediated inflammation leading to cell recruitment and leakage of blood cells. However, it must be pointed out that SPFL is a unique clinical syndrome without parallel with other infectious diseases.

Previous studies on vascular damage in experimental leptospirosis in guinea pigs have demonstrated several degrees of capillary injury and necrosis of endothelial cells.[42] Data on leptospiral load and lung tissue change has been subjective and contradictory due to the methods used for demonstration of leptospiral antigen in hemorrhagic pulmonary parenchyma.[41,43] The presence of antigenic material was found in the lung parenchyma closely associated with areas of tissue changes (Figure 3A). These observations provide a basis for further studies on the role of outer membrane components, putative leptospiral toxins, and the immune response in the pathogenesis of SPFL. The role of acute inflammatory mechanisms is not clear, but most likely involves multiple mediators including the participation of immunoglobulins and complement (Pereira MM and others, unpublished data). It was recently demonstrated that leptospiral major outer membrane components such as lipopolysaccharide and lipoprotein activate macrophages through CD14 and Toll-like receptor 2 (TRL2).[44]

Jaundice in subcutaneous tissue and viscera on gross examination and microscopic lesions in the liver of the marmoset monkeys were seen as early as pid 3. Liver lesions of infected marmoset monkeys and serum levels of bilirubin, AST, and ALT were comparable to those in human leptospirosis. In human leptospirosis, the degree of histopathologic changes found at autopsy by bright-field microscopy is poorly correlated with the degree of functional damage. This also seems to be true for marmoset monkeys.

Leptospira induced different kinds of hepatocytes changes, including hepatocyte condensation, that was often seen, being prominent at pid 6(Figures 1D and 2C). Hepatocytes apoptosis has been previously demonstrated in the early course of leptospirosis in the golden hamster model, with infection being associated with leptospires between liver parenchyma cells.[45] Leptospiral antigens were found mainly within the Kupffer cells in the hepatic sinusoids of infected marmosets, and sometimes associated with necrotic areas as shown by MAbs (Figure 2B). Further study will be required to elucidate if this material demonstrated by MAb F70C24 in tubular cells corresponds to leptospiral antigens or is due to cross-reactivity with cellular components such as cytoskeletal proteins.

In summary, the results for each marmoset monkey at different time points of infection were consistent with the predicted steps based on other animal models and deeply mimic the human pathology. It should be noted that prominent histopathologic changes were consistent with increased antibody levels and the expression of antigens on tissues. Indeed, marmoset monkey (*C. jacchus*) is a suitable experimental model for studies on pathogenesis of the severe pulmonary form of leptospirosis.

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