Pulmonary Disease in Hamsters Infected with *Leptospira interrogans*: Histopathologic Findings and Cytokine mRNA Expressions

Márcia Marinho,* Itamar Souza Oliveira-Júnior, Cristina Maria Rodrigues Monteiro, Silvia Helena Perri, and Reinaldo Salomão

Department of Animal Health and Production, Microbiology Laboratory FOA, Unesp-Estadual Paulista University, Araçatuba, São Paulo, Brazil; Inflammatory Mediators Laboratory, Geriatrics and Gerontology Division, Federal University of São Paulo, Brazil; Infectious Diseases Division, Federal University of São Paulo, Brazil

Abstract. Our aim was to evaluate the pulmonary changes induced by *Leptospira interrogans* infection in hamsters, and the gene expression of endogenous mediators in lung fragments during 28 days of observation. The animals were euthanized on days 4, 7, 14, 21, and 28 post-inoculation. Histopathologic lung analysis showed hemorrhage, pneumonia, alveolar congestion, and infiltrated cellular areas, with increasing severity until day 21 post-inoculation. Tumor necrosis factor (TNF)-α mRNA expression enhanced in first days with peak on day 4 and slightly decreased in the final phase. The interleukin (IL)-10 remained relatively constant throughout the period, with the exceptions of days 4 and 14. The endothelial nitric-oxide synthesis (eNOS) showed an increased expression on day 4, followed by an augment on days 7 and 14, and remaining constant up to day 28 post-infection. Our results demonstrate that inoculation of *L. interrogans* serovar Icterohaemorrhagiae induced pulmonary lesions, including pulmonary hemorrhage, supporting that the lung is a target organ.

INTRODUCTION

Leptospirosis is a globally important zoonotic disease caused by infection with pathogenic *Leptospira* species.¹ It affects humans in all continents, in both rural and urban areas, particularly in developing countries of warm and humid climate.² Vihn and others³ extracted a glycolipoprotein (GLP) present in the cell wall of a strain of *Leptospira interrogans* serovar Copenhageni that presented a cytotoxic effect against L929 fibroblast cells *in vitro*. It was later demonstrated that the GLP induced cytokine production in peripheral blood monocytes from healthy volunteers.⁴ The presence of inflammatory cytokines has also been confirmed in sera of patients with leptospirosis.⁵ Marinho and others⁶ showed that high levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in the supernatant of peritoneal macrophage cellular culture of genetically selected mice inoculated with a sample of *Leptospira interrogans* serovars Canicola was determined by immunoenzymatic assays. The lungs are an important target organ in leptospirosis presenting hemorrhagic pneumonis-tis with varying degrees of severity.⁷,⁸ The pathophysiology of pulmonary injury in leptospirosis is poorly understood. Vascular pulmonary injury is thought to be the outcome of immunologic mechanisms with a disseminated intravascular coagulation-like reaction caused by *Leptospira* toxins.¹⁹ Observation under electronic microscopy revealed that the primary lesion occurs in pulmonary capillary endothelial cells.¹² Andrade and others¹³ concluded that leptospirosis profoundly influences the sodium transport capacity of alveolar epithelial cells and that inadequate handling of pulmonary fluid can affect the pulmonary function, increasing the chance of lung injury. Damage to the pulmonary endothelium occurs without evidence of disseminated intravascular coagulation.¹⁴–¹⁶ Nally and others¹⁵ found that in infected guinea pigs, immunoglobulin C3 were deposited along the alveolar basement membrane in a pattern similar to the one seen in Goodpasture’s syndrome. This finding needs to be confirmed for human leptospirosis, but it is indicative of the underlying autoimmune process observed in the severe pulmonary hemorrhagic syndrome.¹⁶ The role of inflammatory mediators, such as cytokines, in the pathophysiology of sepsis-induced acute lung injury has been extensively studied.¹⁸ We have previously shown that TNF-α and IL-6 were detected in high levels of bronchoalveolar lavage (BAL) in rats injected intraperitoneally with *Escherichia coli*.¹⁹ In this study we aimed to evaluate the expression of tissue TNF-α, IL-10, and endothelial nitric-oxide synthesis (eNOS) mRNA levels in an experimental model of *Leptospira*-induced lung dysfunction, assessed by functional and histologic parameters.

MATERIAL AND METHODS

Animals. The animal experiments were conducted with approval from the Research Ethics Committee of the Federal University of São Paulo (São Paulo, Brazil). Male golden hamsters (*Mesocricetus auratus*), weighing 120 to 150 g, were divided into five groups, each group with six animals. Each experimental set was comprised of a group with five inoculated hamsters and one non-infected hamster per checking point. The hamsters were infected with 10⁵ *Leptospira* of a virulent strain, culture grown in Ellinghausen–McCullough–Johnson–Harris (EMJH) liquid medium (Becton Dickinson, Cockeysville, MD) by intraperitoneal (IP) injection of 1 mL and observed for 4, 7, 14, 21, and 28 days. Negative control animals were injected with 1 mL of EMJH liquid medium alone.

Inoculum. *Leptospira interrogans* serovar Icterohaemorrhagiae (strain n° 11437) was provided by a FIOCRUZ Laboratory, Brazil, and maintained in semi-solid Fletcher medium.

Euthanasia and lung collections. The animals were euthanized at the end of the respective day using a 1 mL/100 g body weight of euthanasia solution sodium pentobarbital (Tiopentax, Cristália, São Paulo, Brazil) by IP injection. At the...
end of the experiment a sternotomy was performed and the lungs and structures were removed. The left lung was divided into two parts, one for histopathology analyses (superior portion of bronchi) and the other for reverse transcription-polymerase chain reaction (RT-PCR) analyses (inferior portion of bronchi). The right lung fragments were washed in phosphate solution (phosphate buffered saline [PBS] 0.1 mol; pH 7.4).

**Histopathology.** The superior portion of the left lungs fragments were dehydrated in graded concentrations of ethanol and then embedded in paraffin. From each lung, 4 µm-thin sections were obtained and stained with hematoxylin and eosin (HE) to evaluate lung morphology. The slides were systematically scanned in a microscope using 100x magnification. All histologic material was coded so that blind examination could be performed by a skilled histopathologist and assigned a score of 0 to 3 crosses (0 = no injury; one cross [+] = slight injury; two crosses [++] = moderate injury; and three crosses [+++] = severe injury) based on the degree of interstitial edema, alveolar edema, hemorrhage, and neutrophil infiltrations.

**Tissue preparation for reverse transcription-polymerase chain reaction.** The other portions of lungs were homogenized and mRNA for TNF-α, IL-10, eNOS, and β-actin (housekeeping gene) expression was performed by RT-PCR. Total RNA was isolated from the lung of 5 animals per group and the RT-PCR was previously described by performed in the lungs according to the following groups: 4, 7, 14, 21, and 28 days. Total RNA was extracted using Trizol reagent according to manufacturer’s protocol (Gibco-BRL, Gaithersburg, MD), and the RNA was ethanol precipitated and dissolved in diethyl pyrocarbonate-treated water and total RNA was quantified spectrophotometrically at 260 and 280 nm (Genesys 5, Spectronic Instruments, Rochester, NY), RNA concentration (1 µg/1 µL). Two micrograms of RNA were reverse transcripted (SuperScript III reverse transcriptase; Invitrogen, Frederick, MD) using oligo(dT) primers. The cDNA was amplified by PCR using specific primers for TNF-α and IL-10, constitutive endothelial NOS (eNOS) and β-actin. The sequences, annealing temperature, and cycles of these primer pairs, 5' and 3', are shown in Table 1. The reverse-transcribed cDNA (0.5 µg in 2 µL) was added with specific cytokine primer pairs to a PCR mix with 1.5 mM MgCl2, 125 mM dNTP, and 2.5 U of Taq DNA polymerase (Invitrogen) in a 20 µL reaction volume. The PCR cycle conditions were performed in a thermal cycler GeneAmp PCR System, model 9700 (Perkin Elmer, Waltham, MA). The PCR products were separated by electrophoresis on 2% agarose gel containing 0.2 µg/mL ethidium bromide. The PCR products were visualized in a transilluminator M26 (BioAgency, SP, Brazil) at 302 nm wavelength. The resulting images were captured (Kodak EDAS 120, NY) and densitometry was performed using an automated gel-imaging system (Image PC, Kodak, NY). The relative amount of inflammatory mediators was expressed by primer densitometry/β-actin.

**RESULTS**

**Morphologic examination.** In general, the pulmonary parenchyma lesions of the hamsters inoculated by intraperitoneal via *L. interrogans* serovar *Icterohaemorrhagiae* presented the same histologic alterations with varying degrees of lesion intensity, from mild to severe (Figure 1A–F and Table 2). On day 4 post-infection, diffuse and discrete areas of focal infiltrate, showing a predominance of neutrophils cells, activated alveolar macrophages were observed, and a discrete area of interstitial pneumonia, showing alveolar congestion and a few hemorrhagic foci, establishing the degree of severity as one cross (+) (Figure 1B) On day 7 post-infection, the inflammatory process had progressed, showing more compromised pulmonary parenchyma lesions, presenting as moderate to severe. At this point, observation revealed the presence of areas of diffuse infiltrate of mononuclear cells, activated alveolar macrophages, and bronchoalveolar epithelial cells, and the presence of neutrophils, albeit rare, constituting chronic interstitial pneumonia associated with hemorrhagic areas (Figure 1C). On day 14 post-inoculation, observation showed deterioration of the inflammatory process presenting areas of extensive infiltrate, predominately mononuclear, including the presence of neutrophils and aggravation of the chronic interstitial pneumonia, with areas of alveolar congestion extending throughout the pulmonary parenchyma. Intense areas of hemorrhaging were observed and the lesion severity was considered accentuated (Figure 1D). On day 21 post-inoculation, the same inflammatory lesions were observed, including areas of chronic interstitial pneumonia showing a high number of activated macrophages. The pulmonary parenchyma tissue was intensively compromised, despite a minor reduction in lesion severity (Figure 1E). On day 28 post-infection, focal areas of chronic interstitial pneumonia with emphysema were observed, accompanied by alveolar congestion and hemorrhaging (Figure 1). Although a reduction in the degree of lesion severity was observed as of day 21 post-inoculation, it was still considered accentuated and quantified as three crosses (+++). It should be highlighted that after day 7 post-infection, the severity of the lung tissue lesions intensified, remaining constant up to day 21, when, curiously, a very high number of activated macrophages was observed, suggesting an attempt to resolve the inflammatory process although severe pulmonary lesions persisted. The lung involvement in leptospirosis is presented as hemorrhagic pneumopathy with diffuse alveolar hemorrhage, (intra-alveolar and interstitial

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>TGAGTCCACCTCTCTCTTCC</td>
<td>57</td>
<td>150</td>
</tr>
<tr>
<td>TNF-α-R</td>
<td>TTGAGAGACATGGGGTGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>GGACAACTACTACTCAGTG</td>
<td>53</td>
<td>214</td>
</tr>
<tr>
<td>IL-10-R</td>
<td>ACAGGGGAGAAATGAGATGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>GGGTCCCTCTCCTTGGGCTGCCACC</td>
<td>63</td>
<td>186</td>
</tr>
<tr>
<td>eNOS-R</td>
<td>GGATCCCTGGGAAAGGGCGGTGAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>CCA ACT GGAGACGAATAAGGAGAGGAC</td>
<td>57</td>
<td>130</td>
</tr>
<tr>
<td>β-actin-R</td>
<td>CAC AAT GCCGATGCATGGAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.** Details of the primers and conditions used for the reverse transcription-polymerase chain reaction (RT-PCR) assays.
areas), including pulmonary edema, fibrin deposition, and capillary lesions characterized by swelling of endothelial cells. However, infiltrations of alveolar spaces by monocytes and neutrophils occurs, inflammatory infiltrates are generally not prominent.

**Cytokines expression in lung tissues.** The TNF-α mRNA expression was very low in control animals (0.03 ± 0.006-fold) compared with infected animals along the 28 days of experiment. Expression of TNF-α mRNA in the days 4 (0.89 ± 0.006-fold), 7 (0.85 ± 0.003-fold), 14 (0.89 ± 0.015-fold) was higher than in the day 28 (0.71 ± 0.07-fold) (Figure 2).

The IL-10 mRNA was elevated in the control group (0.51 ± 0.02-fold) and increased in the days 4 (0.71 ± 0.003-fold), 7 (0.60 ± 0.006-fold), 14 (0.60 ± 0.01-fold), 21 (0.57 ± 0.01-fold), and 28 (0.64 ± 0.02-fold) (Figure 2).

The eNOS gene expression was higher in infected animals than in controls. It showed an increased expression on day 4, followed by an augment on days 7 (0.87 ± 0.06-fold) and 14 (0.93 ± 0.01-fold), remaining constant up to day 28 post-infection (Figure 2).

**DISCUSSION**

Our results demonstrate that intraperitoneal inoculation of *L. interrogans* sorovar Icterohæmorrhagiae in hamsters induced pulmonary dysfunction as a cause of pulmonary hemorrhage that was verified by structural alterations found in histologic exams and detection of TNF-α and eNOS mRNA in lung tissues, supporting that the lung is a target organ for leptospirosis in hamsters. These results underscore our previous report on leptospira-induced lung dysfunctions in those animals, as assessed by gas exchange impairment and alveolar macrophage activity.21

Arean22 and Comby and others23 necropsy reports on cases of fatal human (Weil’s disease) leptospirosis described different degrees of pulmonary compromise according to the lesions presented, particularly characterized by areas of hemorrhaging, edema, capillary congestion, and small foci of leukocytes infiltrate. Leukocytes infiltration, (neutrophils and mononuclear cells on acute and chronic phase, respectively) alveolar congestion, interstitial pneumonia, and hemorrhagic foci were found in the lungs of the hamsters injected with *L. interrogans* in the present study. A time-related severity of the lesions was clearly demonstrated as shown by the increasing severity of the lesions from day 4 to day 21 of infection. Extensive areas of infiltrate and alveolar congestion, as well as of hemorrhaging are found after day 14 of infection. On day 21 post-infection, despite the lesions intensity, some signs of recovery were present and a reduction in the lesions’ severity compared with day 14 was noticed. Of interest, on day 21, an infiltrate of macrophages was present suggesting an attempt to resolve the inflammatory process, although severe pulmonary lesions persisted with alveolar hemorrhage and a few areas with edema. Diffuse alveolar hemorrhage in leptospirosis is likely to occur secondary to a vasculitic process. Although this disease is not listed as a cause of diffuse alveolar hemorrhage

**Table 2**

Pulmonary parenchyma lesions of the hamsters inoculated by intraperitoneal via with *L. interrogans* sorovar Icterohaæmorrhagiae presented histologic alterations with varying degrees of lesion intensity, from mild to severe on day post-inoculation

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Control</th>
<th>4</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Areas of focal infiltrate of neutrophils cells</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Activated alveolar macrophages</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Area of interstitial pneumonia</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Alveolar congestion</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Hemorrhagic foci</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Areas of focal infiltrate of mononuclear cells</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Activated bronchoalveolar epithelial cells</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Score of 0 = no injury, score of + = slight injury, score of ++ = moderate injury, score of +++ = severe injury.
in the review literature, a few reviews suggest that leptospirosis should be considered.\textsuperscript{3,4} However, our results showed that congestion pulmonary and hemorrhage areas were present in histopathologic findings, and there was pulmonary involvement in leptospirosis. Two main mechanisms of leptospirosis pathogenesis are suggested: a toxin-mediated mechanism and/or the immune responses of the host. A toxin-mediated capillary vasculitis is believed to cause lung hemorrhage.\textsuperscript{4}

We aimed to investigate the pathophysiology of Leptospira-lung dysfunctions in hamsters by assessing the gene expression in lung fragments of TNF-\(\alpha\), IL-10, and eNOS. The TNF-\(\alpha\) is a proinflammatory cytokine that presents both protective and damaging consequences for the host.\textsuperscript{18} The TNF-\(\alpha\) production and secretion are regulated by many factors, including interferon (IFN)-\(\alpha\) and IL-10.\textsuperscript{26} The IFN-\(\gamma\) enhances lipopolysaccharide (LPS) induced TNF-\(\alpha\) production, whereas IL-10 is a macrophage deactivator that inhibits TNF-\(\alpha\) production.\textsuperscript{26} The TNF-\(\alpha\) expression in lung tissues was present on day 4 post-infection and remained detected throughout the course of the infection. Only minor detection was found in control animals. In contrast, IL-10 expression shows only a moderate increase in its expression during the course of the disease, mainly because of the constitutive expression found in control animals (Figure 2). A TNF-\(\alpha\) role in the pathophysiology of leptospirosis has been previously documented in experimental and clinical studies. The presence of TNF-\(\alpha\) in the plasma of patients with leptospirosis was associated with infection severity.\textsuperscript{8} Diamant and others\textsuperscript{4} showed that a GLP from \textit{L. interrogans} can induce lymphocyte and monocyte activation \textit{in vitro}, initially stimulating TNF-\(\alpha\) secretion and later IL-10. The presence of TNF-\(\alpha\) mRNA transcription found in lung tissues along with the histopathology findings support a role for this cytokine in this model. Inflammatory biologic activities of TNF-\(\alpha\) have been implicated in the pathogenesis of acute respiratory syndrome, both in experimental and clinical studies.\textsuperscript{27,28} Accordingly, we found high levels of TNF-\(\alpha\) in bronchoalveolar lavage of rats with sepsis and lung disease.\textsuperscript{19} Modulation of IL-10 mRNA expression was much less pronounced than that of TNF-\(\alpha\). In contrast to TNF-\(\alpha\), a constitutive expression of IL-10 mRNA was found in control animals, with only moderate modulation along the 28 days of the experiment. Thus, considering the results obtained in control animals, a clear change in the relative expression of TNF-\(\alpha\) and IL-10 was found during the disease. We have previously reported that the ratios of TNF-\(\alpha\) and IL-10 levels of circulating protein had a good correlation with the disease severity in human leptospirosis, suggesting that an anti-inflammatory response may be protective.\textsuperscript{29}

The eNOS mRNA expression was very low in control animals and presented increased expression on day 4, reaching a peak between day 7 and day 14, remaining as a plateau throughout the entire experiment. The role for endothelial NOS (eNOS) in the pathogenesis of sepsis is unsubstantiated.\textsuperscript{30} Recently, mice overexpressing eNOS were shown to express equivalent levels of iNOS mRNA and to generate levels of plasma NO\textsuperscript{3\textendash} and NO\textsuperscript{\textendash} (an index of iNOS activity) similar to control animals\textsuperscript{31} in response to LPS. However, recently, Connelly and others\textsuperscript{32} demonstrated that eNOS-derived NO plays a key role in facilitating iNOS expression in LPS-induced endotoxaemia in mice \textit{in vivo}. These results inferred the presence of endothelial cell lesions observed in leptospirosis. Endothelial cells, in addition to their involvement in biologic activities that are vital to the organism,\textsuperscript{32} play an important physiologic role: they are targets for local immune and systemic reactions and are involved in the adhesion of neutrophils and monocytes in the induction of endothelial-leukocyte adhesion molecule 1 (ELAM-1) synthesis, in platelet activation factor (PAF) synthesis, in increased synthesis of prostaglandin I\(\textendash\)2 (PGI\(\textendash\)2) secretion, colony-stimulating factor secretion, IL-1 secretion and platelet-derived growth factor (PDGF) secretion.\textsuperscript{33,34} Salles and others\textsuperscript{32} reported that one of the most important alterations in systemic inflammatory response syndrome (SIRS), sepsis, and septic shock is leukocyte adhesion in endothelial cells and that leukocyte adhesion is stimulated by the action of inflammatory cytokines, especially TNF-\(\alpha\) and IL-1.

As outlined, we showed an acute inflammatory response in the lungs of hamsters infected with \textit{L. interrogans}, with pulmonary hemorrhage that varied from moderate to severe, inflammatory cell mobilization, and the presence of mRNA cytokines. The results observed in 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 expression of inflammatory
mediators on tissues. The high expression of eNOS may be related with the action of spirochete or toxin on vascular endothelium. Further understanding of the intrinsic mechanisms related to the immunologic response to leptospirosis could contribute to the development of anti-inflammatory or immunomodulatory therapies that directly interfere in the course of the inflammatory process.

Received August 1, 2008. Accepted for publication December 14, 2008.

Acknowledgements: We thank Márcio A. Custódio for his technical support during the RT-PCR assay, the State of São Paulo Research Foundation (FAPESP; 2205/57085-1), and CNPq (1500057/2005-9) for financial support.

Authors’ addresses: Márcia Marinho, Microbiology Laboratory Estadual Paulista University, Unesp-FOA-Campus Araçatuba, Rua Clôvis Pestana, 793, CEP: 16050-680, Araçatuba, São Paulo, Brazil, Tel: 55-18-3636-1382, Fax: 55(XX)18-3636-1352, E-mail: mmarinho@fmva.unesp.br. Itamar Souza Oliveira-Júnior, Inflammatory Mediators Laboratory, Geriatrics and Gerontology Division, Federal University of São Paulo, Brazil, Tel: 55(XX)11-2347-3581, E-mail: souza.oliveira@unifesp.br. Cristina Maria Rodrigues Monteiro, Department of Animal Health and Production, Estadual Paulista University, Unesp-FOA-Campus de Araçatuba, São Paulo, Brazil, Tel: 55(XX)18-3636-1377, E-mail: monteiro@fmva.unesp.br. Silvia Helena Perri, Department of Animal Health and Production, Estadual Paulista University, Unesp-FOA-Campus Araçatuba, São Paulo, Brazil, Tel: 55(XX)18-3636-1377, E-mail: shvperri@fmva.unesp.br. Reinaldo Salomão, Infectious Diseases Division, Federal University of São Paulo, Brazil, Tel: 55(XX)11-5084-4262, E-mail: rsalomao@unifesp.br.

REFERENCES
