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Attenuated Nephritis in Inducible Nitric Oxide Synthase Knockout C57BL/6 Mice and Pulmonary Hemorrhage in CB17 SCID and Recombination Activating Gene 1 Knockout C57BL/6 Mice Infected with *Leptospira interrogans*[▽]

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The aims of this study were to investigate the frequency of pulmonary hemorrhage (PH) in mice unable to produce functional B and T lymphocytes and to explore the effect of an inducible nitric oxide synthase gene (*Inos*) knockout (KO) on the frequency/severity of interstitial nephritis *in vivo*. We studied the outcome of infection by the virulent *Leptospira interrogans* serovar Copenhageni strain Cop. The animals used were *Inos* KO mice, recombination activating gene 1 (*Rag1*) KO mice, CB17 severe combined immunodeficiency (SCID) mice, and the respective wild-type (WT) C57BL/6 and BALB/c controls. The *Inos* KO and WT mice survived with no clinical symptoms of leptospirosis. The frequency and severity of nephritis was significantly lower in the *Inos* KO mice. All of the *Rag1* KO and SCID animals died of acute leptospirosis, whereas all of the WT mice survived. PH was observed in 57 and 94% of *Rag1* KO mice and in 83 and 100% of SCID mice, using inoculum doses of 10^7 and 10^6 leptospires, respectively. There was no evidence of PH in the WT controls. In conclusion, the loss of the *Inos* gene had a negligible effect on the outcome of leptospiral infection, although we observed a reduced susceptibility for interstitial nephritis in this group. Of note, the absence of functional B- and T-cell lymphocytes did not preclude the occurrence of PH. These data provide evidence that PH in leptospirosis may not be related only to autoimmune mechanisms.

Leptospirosis is a zoonosis with a wide clinical spectrum that includes fatal outcomes due to acute renal failure and pulmonary hemorrhage (PH). Pathogenic leptospires are carried by diverse mammalian reservoirs, and peridomiciliary rodents are the most important source of infection in urban settings (1). Major efforts in vaccine development and basic research on mechanisms of disease have been carried out in recent years; however, our knowledge of the genetic determinants involved in host protection and pathogenesis remains limited (10).

Among the diverse animal models used in leptospirosis research, guinea pigs and hamsters are the most suitable laboratory rodents for reproducing acute lethal infection (12, 16, 17). Rats are resistant to acute disease and are more suited to studies focusing on mechanisms of persistent infection (3, 13). The mouse model offers a broad array of immunological and genetic tools available for basic research; however, it has been poorly explored in leptospirosis. In previous reports, we described differences in the outcome of experimental leptospiral infection among distinct wild-type (WT) mouse strains (15) and the lack of significant effects on outcome of knockouts (KO) in the genes for tumor necrosis factor alpha receptor Rp55, gamma interferon, and interleukin 4 (2).

A potential role for autoimmunity in leptospirosis-associated PH was suggested based on observations in the guinea pig model of leptospirosis (12) and, to a lesser extent, in human patients with severe pulmonary hemorrhage syndrome (7). However, the involvement of auto-antibodies in PH was countered by a description of lethal PH in experimentally infected severe combined immunodeficiency (SCID) mice lacking functional B- and T-lymphocyte subsets (19). Rats are the prototype model of resistance to acute lethal infection (3), but consistent with the observation from SCID mice, rats treated with cyclophosphamide (which suppresses humoral immunity) develop PH (18). However, the observation of PH in SCID mice is not reliable because it was observed in the C3H/HeJ mouse strain background (19) but not the C3H background (14). In this study, we reproduced these experiments in the following murine models: CB17 SCID and C57BL/6 recombination activating gene 1 (*Rag1*) KO mice. CB17 SCID mice are unable to produce functional B and T lymphocytes due to a mutation in the *Prkdc* gene, which encodes a DNA-dependent protein kinase involved in DNA double-strand break repair and recombination. The strain is similar to the BALB/c strain except that it carries the *Igh-1b* allele from the C57BL/Ka strain. *Rag1* KO mice lack a gene that plays an important role in the rearrangement and recombination of the genes of immunoglobulin and T-lymphocyte receptor molecules during the process of VDJ recombination. Thus, *Rag1* KO mice are unable to generate specific B and T lymphocytes. Mutations in both *Prkdc* and *Rag-1* genes are listed as causes of human SCID. In

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TABLE 1. Evaluation of leptospirosis in iNOS-deficient mice infected with *L. interrogans* strain Cop

Mouse strain[a]	Expt	Size of inoculum	% of mice with nephritis[b] (no. with nephritis/total no.)		Leptospiral load [median (IQR)[c]]	MAT titer [median (IQR)]
			Positive	Severe		
Inos KO	1	10^3	57.1 (4/7)	28.6 (2/7)	10 (4.5)	400 (300)
WT			86.7 (13/15)	33.3 (5/15)	5 (7.5)	300 (200)
Inos KO	1	10^6	25.0 (2/8)	12.5 (1/8)	0 (2.5)	400 (350)
WT			73.3 (11/15)	40.0 (6/15)	3 (7.5)	400 (1,200)
Inos KO	2	10^6	33.3 (5/15)[d]	13.3 (2/15)	0 (4.0)	800 (800)
WT			80.0 (12/15)	40.0 (6/15)	3 (8.5)	800 (1,400)
Inos KO	3	10^6	40.0 (6/15)	20.0 (3/15)	4 (14)[d]	400 (600)
WT			66.7 (10/15)	26.7 (4/15)	1 (1.0)	400 (700)
Inos KO	Total	10^6	34.2 (13/38)[d]	15.8 (6/38)[d]	8.1 ± 17.9[e]	400 ± 600[e]
WT			73.3 (33/45)	35.6 (15/45)	7.6 ± 18.8[e]	400 ± 1,400[e]

[a] *Inos* KO, iNOS gene-deficient murine strain; WT, C57BL/6 wild-type control.
[b] Positive, grade + or higher nephritis; Severe, grade ++ or +++ nephritis.
[c] IQR, interquartile range.
[d] $P < 0.05$ compared to the control group.
[e] Median ± standard deviation.

our previous report, the C57BL/6 background mice exhibited high leptospiral loads in kidney samples and developed severe inflammatory lesions, while these features were not observed in the BALB/c mice (15).

We have also reported the association between high serum levels of nitric oxide (NO) and the severity of renal involvement in patients with severe leptospirosis (9) Renal production of NO could be involved in transport defects in renal tubular cells (4). *In vitro* studies have previously reported the activation of a broad range of inflammatory genes, such as those for transcription factor NF-κB, inducible nitric oxide synthase (iNOS), monocyte chemotactic protein-1, and tumor necrosis factor alpha, by renal tubular cells in response to exposure to leptospire-derived products. These findings have been interpreted as a molecular trigger for interstitial nephritis (20–22). The genetic deficiency of iNOS has not been investigated *in vivo*. Nitric oxide secreted during an immune response acts as a free radical and generates toxic products against bacteria. Thus, in theory, the genetic deficiency of iNOS could alternatively promote higher loads of leptospires in blood and tissues or result in less severe inflammatory lesions in kidneys.

The aims of this study are as follows: (i) to investigate the frequency of PH in mice unable to produce functional B and T lymphocytes in light of the hypothesis that PH in leptospirosis is related to immunopathogenesis/auto-antibodies and previous unreliable data on the frequency of this complication in SCID mice of the C3H and C3H/HeJ backgrounds and (ii) to explore the effect of iNOS gene (*Inos*) KO on the frequency and severity of interstitial nephritis *in vivo* in light of previous *in vitro* data suggesting that leptospiral products induce renal tubular cells to express proinflammatory genes, such as *Inos*.

MATERIALS AND METHODS

***Leptospira* strains and culture conditions.** *L. interrogans* serovar Copenhageni strain Cop was cultivated in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) modified Tween 80-bovine albumin medium (Difco Laboratories) at 29°C, and leptospires were counted in a Petroff-Hausser counting chamber (Fisher Scientific). This strain was passaged and reisolated from hamsters four times and stored at −70°C. Frozen aliquots were thawed and passaged in liquid medium 14 times prior to use as a low-passage-number isolate in the infection experiments. The virulence of this strain was evaluated in hamsters as described previously, and the 50% lethal dose (LD_{50}) was calculated to be ~164 leptospires (15).

Experimental murine model of leptospirosis. The murine strains used in this study were C57BL/6 *Inos* KO (B6.129P2-Nos), C57BL/6 *Rag1* KO [129S(Cg)-Rag1], CB17 SCID, and the respective C57BL/6 and BALB/c WT controls. All mouse strains were purchased from The Jackson Laboratory and maintained in the animal unit at Fiocruz-BA. Animals were monitored daily for clinical signs of disease (loss of activity, jaundice, external hemorrhage, and moribund state). The *Inos* KO and the WT control mice (7 to 15 per group) were inoculated by intraperitoneal injection (10^3 and 10^6 leptospires in 1 ml phosphate-buffered saline [PBS]) in one experiment. The following endpoints of infection were evaluated: survival, renal pathology, leptospiral load, and immune response (Table 1). The immunodeficient mice and the WT controls (5 to 15 per group) were inoculated by intraperitoneal injection (10^6 and 10^7 leptospires in 1 ml PBS) in one experiment. The following endpoints of infection were evaluated: survival, time between infection and death, and the frequency of gross pulmonary hemorrhage (Table 2). The Ethics Committee of the Oswaldo Cruz Foundation approved all animal protocols used in this study.

Gross pathology and light microscopy. Animals presenting a moribund state were euthanized immediately, and convalescent survivors were euthanized 28 days postinfection. Necropsies were performed immediately after euthanasia. At necropsy, lungs were examined to detect macroscopic PH. Only macroscopic hemorrhages were reported as PH for purposes of this analysis. In all cases, microscopic examination was performed to confirm the presence of massive alveolar hemorrhaging. One kidney was fixed in 4% formalin and embedded in paraffin, and 4- to 5-μm-thick sections were used for conventional histology. A semiquantitative estimation of interstitial nephritis was used as previously described (2). Briefly, in grade + nephritis, infiltrate was rich in macrophages and lymphocytes and restricted to periarterial areas; in grade ++ nephritis, infiltrate extended to other renal parenchymal zones with 1 to 2 lesions per field of view at ×100 magnification; and in grade +++ nephritis, lesions were detected in more than 2 areas per field of view at ×100 magnification. For purposes of this analysis, grades ++ and +++ were considered to be severe nephritis.

Imprint detection of leptospires. Imprints were obtained by direct pressure of the cut surface of the tissue sample onto poly-L-lysine-coated glass slides, and leptospires were visualized by immunofluorescence as described previously (5). The immunofluorescence-based leptospiral detection in imprint samples is the detection method of choice in our laboratory, as it has proved to be reliable and has the advantages of simplicity and reduced time to result compared to immunofluorescence in frozen sections. Importantly, while renal colonization may lead to crowding of leptospires in tubular lumens, imprint-based visualization easily identifies isolated leptospires and, thus, has the additional advantage of allowing

TABLE 2. Lethal outcome, days between infection and death, and frequency of macroscopic pulmonary hemorrhages in immunodeficient and immunocompetent mice of the correspondent background

Expt[a]	Size of inoculum	No. of deaths/ total no. of mice (%)	No. of days between infection and death [median (IQR)][b]	No. of mice with macroscopic PH/ total no. (%)
1	10^7			
Rag1 KO		7/7 (100)	9 (0.5)	4/7 (57)
B6		0/15	NA	0/15
CB17 SCID		12/12	7 (0)	10/12 (83)
BALB/c		0/15	NA	0/15
2	10^6			
Rag1 KO		5/5 (100)	11 (0)	5/5 (100)
B6		0/15	NA	0/15
CB17 SCID		15/15 (100)	10 (0)	15/15 (100)
BALB/c		0/10	NA	0/10
3	10^6			
Rag1 KO		12/12 (100)	9 (0)	11/12 (91)
B6		0/14	NA	0/14
CB17 SCID		15/15 (100)	10 (0)	15/15 (100)
BALB/c		0/10	NA	0/10
Total	10^6			
Rag1 KO		17/17 (100)	9 (2)	16/17 (94)
B6		0/29	NA	0/29
CB17 SCID		30/30 (100)	10 (0)	30/30 (100)
BALB/c		0/10	NA	0/10

[a] *Rag1* KO, recombination activating gene 1 knockout mice; B6, C57BL/6 strain; CB17 SCID, CB17 mice with severe combined immunodeficiency.
[b] IQR, interquartile range; NA, not applicable.

easier quantification. In a previous study, we used this assay to quantify leptospiral density in murine kidney samples (15). Leptospires were quantified in kidney imprints, and the results expressed as the mean value for 10 fields of view at ×400 magnification. Only easily identifiable, intact, spiral-shaped organisms were included. Imprint samples of lung and liver from animals that developed acute lethal disease were analyzed.

Serology assays. The microscopic agglutination test (MAT) was performed as described previously, except that only the *L. interrogans* serovar Copenhageni strain Cop was used as the live antigen (15). An in-house anti-*Leptospira* IgG enzyme-linked immunosorbent assay (ELISA) was performed as previously described (15).

Statistics. Statistical analyses and graphical presentation of the data were performed using the Prism version 4.03 software package (Graph Pad). Categorical data were compared by Fisher's exact test, and numerical data were compared by the nonparametric Mann-Whitney test; a P value of <0.05 was considered significant.

RESULTS

***Inos* gene-deficient murine model of leptospirosis.** Both *Inos* KO and WT mice, regardless of inoculum dose, survived with no clinical symptoms of leptospirosis. Furthermore, there were no significant differences between the reciprocal MAT titers for specific anti-*Leptospira* agglutinating antibodies or IgG antibodies in either group (Table 1 and Fig. 1). The data on renal pathology, the leptospiral load in kidney samples, and the MAT reciprocal titer are summarized in Table 1. Overall, the leptospiral load was slightly higher, but not significantly so, in kidney samples from the *Inos* KO mice than in kidney samples from the WT controls. Of note, in the third experiment (10^6 leptospires), a significantly higher leptospiral load was observed in the *Inos* KO group. The results from the three experiments at the 10^6 inoculum showed that the *Inos* KO mice were significantly less susceptible to interstitial nephritis (grade +) than the WT group (34 versus 73%, respectively; $P < 0.01$) and, particularly, were less susceptible to severe nephritis (16 versus 36%, respectively; $P < 0.001$).

B- and T-lymphocyte-deficient murine model of leptospirosis. Both the CB17 SCID and *Rag1* KO murine strains were highly susceptible to acute lethal leptospirosis (Table 2). The median interval from infection to death was 9 days in *Rag1* KO mice, regardless of the inoculum dose. The median intervals to death for infected CB17 SCID mice were 7 and 10 days for inoculum doses of 10^7 and 10^6 leptospires, respectively. All animals developed severe jaundice and presented typical target organ pathology, including acute tubular damage and detrabeculation of hepatocytes (Fig. 2). The infected WT controls survived until 28 days postinfection, with no symptoms of leptospirosis.

Overall, macroscopic PH was observed in 57 and 94% of *Rag1* KO mice infected with 10^7 and 10^6 leptospires, respectively. PH lesions were observed in 82 and 100% of the CB17 SCID mice infected with 10^7 and 10^6 leptospires, respectively. When PH was noted macroscopically, microscopic evaluation was used to confirm the presence of massive recent intraalveolar hemorrhaging (Fig. 2). The quantification of leptospires in the target organs of the immunodeficient mice found high loads of leptospires in all groups and experiments (Table 3). The leptospiral loads of the immunodeficient mice and WT controls were not compared because the immunodeficient mice died 7 to 10 days postinfection, while the WT mice survived and were only examined on day 28 postinfection. Thus, differences in leptospiral load could be attributed to the time point of infection (acute lethal disease versus convalescence) rather than the effect of immune status.

DISCUSSION

The loss of the *Inos* gene in mice had no apparent effect on their survival or development of agglutinating or specific IgG antibodies against *Leptospira*. In theory, impaired NO production during the immune response to leptospirosis could be

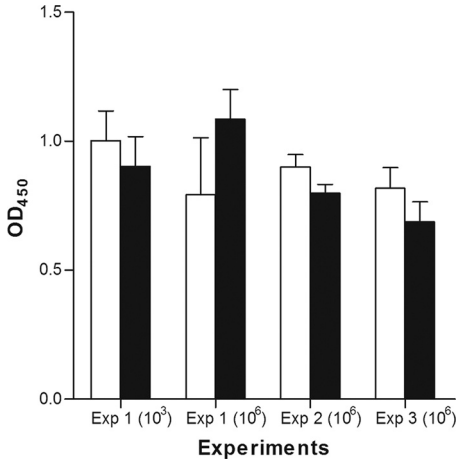

FIG. 1. ELISA analysis of serum anti-leptospiral IgG levels in infected mice 28 days postinfection. The graph compares antibody levels in C57BL/6 wild-type mice (solid bars) and inducible nitric oxide synthase knockout mice (open bars). The error bars represent the standard error of the mean for each group. OD_{450}, optical density at 450 nm.

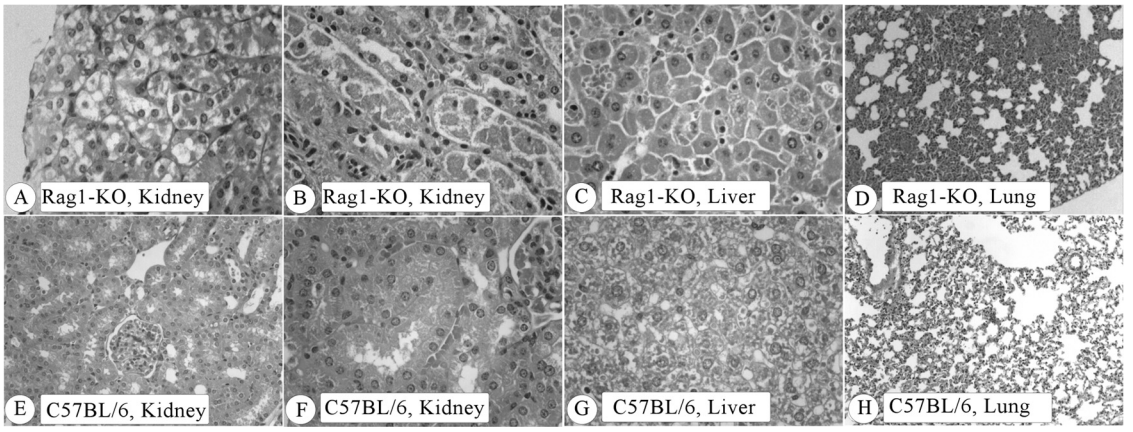

FIG. 2. Typical lesions of leptospirosis in a recombination activating gene 1 knockout C57BL/6 mouse that died 9 days after infection with strain Cop at a 10^6 inoculum. All tissue samples are stained with hematoxylin and eosin. (A) Marked cell swelling of epithelial cells of proximal tubules (×400). (B) Advanced necrosis of proximal tubules (×400). (C) Detrabeculation of hepatocytes (×400). (D) Microscopic foci of a pulmonary hemorrhage (×100). (E to H) For purposes of comparison, photomicrographs of tissue samples from a wild-type C57BL/6 mouse with no lesions are shown. (E and F) Kidney (×200 and ×400, respectively). (G) Liver (×400). (H) Lung (×100).

related to the slightly higher loads of leptospires observed in the tissue samples. However, the significantly higher leptospiral load observed was not reproducible (Table 1). The only significant difference observed between the *Inos* KO and the WT C57BL/6 mice was that the transgenic animals did not develop interstitial nephritis to the same degree or severity as the WT control. This result is in accordance with the hypothesis that the expression of proinflammatory markers by renal tubular cells *in vitro* after exposure to leptospiral products may be related to the development of interstitial nephritis *in vivo* (20–22).

The present study confirms previous reports, using mice of other backgrounds (C3H and C3H/HeJ), that immunodeficiency results in high susceptibility to acute infection, rapidly progressing to death (14, 19). In addition, immunodeficiency resulted in high loads of leptospires in the target organs, as seen upon necropsy, in accordance with a previous report on C3H/SCID mice (14). We observed the typical target organ pathology associated with leptospirosis, including acute tubular damage and detrabeculation of hepatocytes, similar to the pathology described in humans and in other models of lethal leptospirosis, such as hamsters (16).

TABLE 3. Quantification of leptospires in target organs of immunodeficient mice at necropsy

Mouse strain[a]	Expt	Size of inoculum	Median no. of leptospires (IQR[b]) in:		
			Kidney	Liver	Lung
Rag1 KO	1	10^7	154 (29)	60 (53)	49 (33)
CB17 SCID			26 (20)	20.5 (27.5)	2 (3.5)
Rag1 KO	2	10^6	220 (41)	258 (29)	159 (17)
CB17 SCID			141 (37)	181 (32)	155 (28.5)
Rag1 KO	3	10^6	29 (33)	24 (32)	29 (32.5)
CB17 SCID			160 (48)	143 (47.5)	123 (40.5)

[a] *Rag1* KO, recombination activating gene 1-deficient C57BL/6 strain; CB17 SCID, CB17 severe combined immunodeficiency strain.
[b] IQR, interquartile range.

There are insufficient data to attribute the pathogenesis of leptospirosis-related PH to a single mechanism. Furthermore, it is reasonable to assume that the severe pulmonary forms result from a multifactorial response to the direct toxic effects of exposure to leptospires, the effects of systemic inflammation on the alveolar wall, hemostatic disorders, and uremia (11). Nally and colleagues described a linear deposition of antibodies and complement in the guinea pig model, suggesting a potential role for autoantibodies in the pathogenesis of leptospirosis-associated PH (12). This mechanism associates leptospirosis-associated pulmonary disease with Goodpasture's syndrome, where autoantibodies against the glomerular basement membrane (GBM) cross-react with the alveolar septal matrix, causing massive alveolar hemorrhaging. However, the original evaluation of serum anti-GBM antibodies in leptospirosis patients with and without PH found no association between anti-GBM antibodies and lung disease. There was no difference in serum anti-GBM antibody levels between patient and control groups for either acute-phase or convalescent-phase sera (8). In addition, Craig and colleagues found no evidence for anti-GBM antibodies in 40 leptospirosis patients (6). In the present study, mice that were unable to produce functional B and T lymphocytes developed severe PH. This finding suggests that autoimmunity is not a major mechanism for PH in experimental leptospirosis, at least in the murine model and/or in *L. interrogans* serovar Copenhageni infections.

Conclusion. The absence of a functional *Inos* gene in the murine model had a minimal effect on the outcome of leptospiral infection, except for a significantly reduced susceptibility to the development of interstitial nephritis. The absence of functional B and T lymphocytes does not preclude the occurrence of PH. These data provide strong evidence that PH in leptospirosis is not related only to autoimmune mechanisms.

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