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Leptospirose Experimental em Camundongos Imunodeficientes e Camundongos Deficientes para Enzima Óxido Nítrico Sintase Induzível

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Dissertação apresentada como requisito para a obtenção do grau de Mestre em Biotecnologia.

Orientador: Prof. Dr. Daniel A. Athanazio

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"Leptospirose Experimental em Camundongos Imunodeficientes e Camundongos Deficientes para Enzima Óxido Nítrico Sintase Induzível"

Elaborada por:

MAURÍCIO DE SOUSA BANDEIRA

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Resumo

Estudamos a evolução da infecção pela *Leptospira interrogans* sorovar Copenhageni cepa Cop. Os animais utilizados foram camundongos óxido nítrico sintase induzível (iNOS) *Knock Out* (KO), camundongos gene ativador de recombinação 1 (RAG1) KO, camundongos CB17 imunodeficiência combinada grave (SCID), e os respectivos controles do tipo selvagem C57BL / 6 e BALB / c. Os camundongos iNOS KO e os controles do tipo selvagem sobreviveram sem sintomas clínicos de leptospirose. A frequência e gravidade de nefrite foi significativamente menor nos camundongos iNOS KO. Todos os animais RAG1 KO e SCID morreram de leptospirose aguda, enquanto que todos os camundongos controles do tipo selvagem sobreviveram. A hemorragia pulmonar (HP) foi observada em 57 e 94% dos camundongos RAG1 KO e em 83 e 100% dos camundongos SCID, utilizando doses de inóculos de 10^7 e 10^6 leptospiras, respectivamente. Não houve evidência de HP nos controles do tipo selvagem. Em conclusão, a perda do gene iNOS teve um efeito insignificante sobre o resultado da leptospirose, embora observou-se uma suscetibilidade reduzida para a nefrite intersticial neste grupo. De nota, a ausência de linfócitos B e T funcionais não impede a ocorrência de HP. Estes dados fornecem evidência de que a HP na leptospirose pode estar relacionada apenas aos mecanismos autoimunes.

Palavras-chave: *Leptospira*; leptospirose; camundongos; hemorragia pulmonar.

Abstract

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 inoculums 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.

Keyword: *Leptospira*; leptospirosis; mice; pulmonary hemorrhage.

Lista de Abreviaturas e Siglas

iNOS	Óxido Nítrico Sintase Induzível
IgG	Imunoglobulina tipo G
RAG-1	Gene Ativador de Recombinação 1
SCID	Imunodeficiência Combinada Grave
KO	*Knock Out*
WT	*Wild type*
PH	*Pulmonary Hemorrhage*
WHO	*World Health Organization*
SPHS	Síndrome Hemorrágica Pulmonar Grave
SARA	Síndrome da Angústia Respiratória do Adulto
GBM	Membrana Basal Glomerular
PCR	Reação em cadeia de polimerase
DNA	Ácido Desoxirribonucléico
NO	Óxido Nítrico

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1. Introdução

A leptospirose é uma zoonose de distribuição mundial causada por espiroquetas patogênicas do gênero *Leptospira* spp, transmitidas através do contato direto com a urina de animais portadores ou indiretamente pela exposição à água contaminada (ADLER e DE LA PEÑA MOCTEZUMA, 2009; BHARTI et al., 2003; WHO, 2003; FAINE et al., 1999). Historicamente, a leptospirose é uma doença endêmica confinada a um ambiente rural, mas que ao longo do século XX passou a causar epidemias anuais em ambientes urbanos associadas às estações chuvosas e enchentes nos países em desenvolvimento com saneamento precário (MCBRIDE et al., 2005; BHARTI et al., 2003; KO et al., 1999; FAINE et al., 1999). Nos países desenvolvidos, a incidência aumenta em associação a pratica de esportes aquáticos e atividades recreacionais com contato com vida silvestre ou lagos (MCBRIDE et al., 2005).

A infecção humana tem um amplo espectro clínico: frequentemente tem curso assintomático apresentando formas graves em 5-15% dos casos, como a tríade de Weil caracterizada por icterícia, insuficiência renal e diátese hemorrágica (10 a 15% de letalidade na maioria das séries); e formas graves de envolvimento pulmonar tais como a síndrome hemorrágica pulmonar grave (SPHS), que pode se apresentar como síndrome da angústia respiratória do adulto (SARA) ou hemorragia pulmonar maciça, com letalidade superior a 50% em muitas séries (MEDEIROS et al., 2010; MCBRIDE et al., 2005; BHARTI et al., 2003).

Diversos modelos animais têm sido utilizados aos longos dos anos para a reprodução da leptospirose, tais como cães, cobaias, hamsters, gerbils e primatas não humanos, que são capazes de reproduzir os achados da doença humana (DE BRITO et al., 1966; NAVARRO e KOCIBA, 1982; OLIVA et al., 1994; YUKAWA et al., 1994; PEREIRA et al., 2005). Cobaias e hamsters são os roedores de laboratório mais usados para a reprodução da infecção aguda letal, pois desenvolvem lesões em órgãos alvos semelhantes às observadas em humanos, sendo amplamente usados em estudos que envolvem patogênese e testes de novos tratamentos e

vacinas (FAINE et al.,1999; NALLY et al., 2004; SILVA et al., 2008; SPICHLER et al., 2007). Os ratos representam o protótipo de resistência à doença aguda e um modelo potencial para a infecção persistente sem manifestações clínicas (ATHANAZIO et al., 2008; NALLY et al., 2005a). O modelo de camundongos apresenta uma relativa variabilidade relacionada a desfecho da infecção devido a linhagens diferentes, idade e o tamanho do inóculo (FAINE, 1962), entretanto o modelo murino tem a vantagem de uma ampla gama de ferramentas imunológicas e genéticas disponíveis para a pesquisa básica, entretanto, tem sido pouco explorado na investigação da leptospirose.

Estudos recentes utilizam camundongos mutantes ou transgênicos para avaliação do papel de determinados genes nos mecanismos imunológicos relacionados à leptospirose experimental (NALLY et al., 2005b; VIRIYAKOSOL et al., 2006; ATHANAZIO et al., 2008; CHASSIN et al., 2009), mas apenas recentemente estes animais passaram a ser utilizados na pesquisa aplicada à leptospirose. Estudos em animais mutantes com defeitos genéticos específicos ou transgênicos começaram a ser desenvolvidos, embora a susceptibilidade/resistência à leptospirose de animais do mesmo *background* não sejam conhecidas (NALLY et al., 2005b; PEREIRA et al., 1998; VIRIYAKOSOL et al., 2006). Recentemente demonstramos que as linhagens murinas mais amplamente usadas em laboratório (A, CBA, BALB/c e C57Bl/6), são resistentes à doença aguda letal, mas desfechos secundários tais como frequência de nefrite intersticial, carga renal de *Leptospira* e produção de anticorpos são diferentes a depender do genótipo murino (SANTOS et al., 2010) (ANEXO I).

O óxido nítrico (NO) é um radical livre de vida curta sintetizado a partir da L-arginina pela reação catalítica do óxido nítrico sintase (NOS). As isoformas de NO de mamíferos incluem: duas enzimas expressas constitutivamente (cNOS), as neuronais (nNOS) e endotelial (eNOS) e uma isoforma NOS induzível ou iNOS (ALDERTON et al., 2001; FLORA FILHO e ZILBERSTEIN, 2000). As isoformas cNOS são reguladas a nível pós - traducional, enquanto a isoforma iNOS é regulada primariamente por taxa de transcrição (STUEHR, 1999; AKTAN, 2004). Em baixas concentrações o NO é um mediador multitarefa biológico (vasodilatador e microbicida) no organismo vivo, e em altas concentrações o NO pode ser citotóxico (KLEINERT et al., 2004).

A patologia renal na leptospirose é tradicionalmente descrita como uma combinação entre dano agudo tubular com nefrite intersticial (FAINE et al., 1999) sendo que a inflamação aparentemente é um evento tardio no envolvimento renal (AREAN, 1962). Se a inflamação renal é um evento tardio secundário ao dano tóxico tubular, esta observação limita a interpretação de um grande número de estudos recentes que focam na indução de expressão de mediadores pró-inflamatórios nas células renais *in vitro* por produtos de leptospiras (YANG et al., 2006; YANG et al., 2000). A expressão de genes pró-inflamatórios como o da enzima óxido nítrico sintase induzível (iNOS) pode ser de importância menor pela sua associação proposta com a nefrite intersticial observada na leptospirose (YANG et al., 2006; YANG et al., 2002), mas por seu efeito no transporte tubular visto que o óxido nítrico é um reconhecido inibidor fisiológico do cotransportador de Na, K, 2 Cl (CERQUEIRA et al., 2008). Um complicador adicional para interpretação destes estudos *in vitro* é que vários deles usam células murinas e não é claro até que ponto diferentes linhagens murinas realmente desenvolvem nefrite por leptospirose *in vivo* (ATHANAZIO et al., 2008). A correlação entre a expressão de iNOS no tecido renal e as alterações histopatológicas não foi explorada na leptospirose.

A presença de hemorragia pulmonar na leptospirose é descrita há décadas em países como China e Coréia (FAINE et al., 1999; PARK et al., 1989), no entanto, uma epidemia rural de leptospirose na Nicarágua, em 1995, associada à alta frequência de hemorragia pulmonar chamou atenção da comunidade internacional (TREVEJO et al., 1998). Desde então, a hemorragia pulmonar associada à leptospirose passou a ser relatada em diversas regiões do mundo onde antes não era detectada. Em Salvador, os primeiros casos foram detectados em 2003 e, em 2005, já representavam a principal causa de óbito (GOUVEIA et al., 2008).

A frequência de hemorragia pulmonar vem aumentando em diferentes áreas geográficas do mundo, no entanto, não existe uma explicação clara para esta tendência. A patogênese da hemorragia pulmonar associada à leptospirose ainda não é bem compreendida e pesquisas recentes indicam um papel potencial da autoimunidade e/ou mecanismos multifatoriais.

Camundongos imunodeficientes (SCID) apresentam maior suscetibilidade à doença grave após infecção por leptospiras patogênicas conforme dados prévios

obtidos nas linhagens C3H/HeJ-SCID (VIRIYAKOSOL et al., 2006) e C3H/SCID (NALLY et al., 2005b). Entretanto, estes dois estudos mostram dados conflitantes quanto à presença de hemorragia pulmonar nas formas graves de infecção. Os camundongos C3H/HeJ-SCID desenvolvem hemorragia pulmonar, enquanto a linhagem C3H/SCID não. Estes dados são relevantes visto que a presença de hemorragia pulmonar em camundongos SCID é uma forte evidência contra a hipótese recente de um mecanismo de imunopatogênese / auto-imunidade, conforme sugerido no modelo de hamsters (NALLY et al., 2004). O depósito linear de anticorpo e complemento ao longo dos septos alveolares observado em cobaias sugere uma patogênese similar à síndrome de Goodpasture, na qual autoanticorpos contra o colágeno IV podem causar um quadro grave de glomerulonefrite e hemorragias pulmonares maciças (NALLY et al., 2004). Um estudo baseado em imunofluorescência realizado em 17 necropsias de pacientes com leptospirose de São Paulo demonstrou padrão linear de depósito de anticorpos em 12 casos (71%) (CRODA et al., 2010).

2. Justificativa

O modelo murino é pouco explorado na leptospirose embora seu uso apresente a vantagem da ampla facilidade na obtenção de animais transgênicos com deficiências genéticas específicas ou de anticorpos monoclonais contra diversas moléculas e vias de interesse em pesquisa básica. O uso do camundongo na leptospirose experimental é limitado pela observação de que estes animais não representam nem o extremo de susceptibilidade à doença aguda letal, como hamsters e cobaias, nem o extremo de resistência à doença, como ratos.

O uso de modelo de camundongos transgênicos (no caso dos animais *knock-out* para iNOS) ou para camundongos mutantes (no caso doa animais SCID) foi pouco explorado na pesquisa básica em leptospirose. A observação do efeito da deficiência da enzima Óxido Nítrico Sintase Induzível *in vivo* é um passo importante na compreensão da relação entre este gene e a nefrite intersticial observada na leptospirose, o que até agora esta limitado a estudos *in vitro*.

O uso de modelo animal de imunodeficiência é importante para testar a hipótese de que o quadro de hemorragia pulmonar, cuja patogenia é obscura, pode ter a auto-imunidade como mecanismo preponderante. Não foi demonstrado até agora, em modelo experimental ou em pacientes, a presença de auto-anticorpos relacionados com formas pulmonares graves na leptospirose.

3. Objetivos

3.1. Objetivos gerais

Investigar diferenças no desfecho da infecção em camundongos selvagens, camundongos transgênicos *knock-out* para o gene da enzima Óxido Nítrico Sintase Induzível e camundongos imunodeficientes (SCID).

3.2. Objetivos específicos

1. Investigar diferenças no desfecho (sobrevida, resposta imune e carga de leptospiras) da infecção em camundongos selvagens ou deficientes para o gene da enzima Óxido Nítrico Sintase Induzível (iNOS).

2. Investigar diferenças no desfecho (sobrevida, carga de leptospiras e frequência de hemorragia pulmonar) da infecção em camundongos imunodeficientes para células B e T (animas SCID) e nos controles selvagens.

4. Metodologia e Resultados

4.1. Artigo Científico Publicado

BANDEIRA, M., SANTOS, C.S., DE AZEVEDO, E.C., SOARES, L.M., MACEDO, J.O., MARCHI, S., DA SILVA, C.L.R., CHAGAS-JUNIOR, A.D., MCBRIDE, A.J., MCBRIDE, F.W., REIS, M.G., ATHANAZIO, D.A. 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*.

Infect Immun 79: 2936–2940. 2011.

Neste artigo, comparamos os desfechos da infecção por *Leptospira interrogans* sorovar Copenhageni cepa Cop em camundongos C57BL/6 selvagens e transgênicos *knock-out* para o gene da enzima Óxido Nítrico Sintase induzível (iNOS). Ambos os grupos sobrevivem sem sintomas e as dosagens de anticorpos aglutinantes e anticorpos IgG específicos anti-*Leptospira*, assim como a quantificação de leptospiras nos rins são similares nos dois grupos. Os animais sem a enzima iNOS, no entanto, desenvolvem nefrite intersticial com menor freqüência e com formas mais leves. O estudo avaliou ainda o desfecho da infecção pela mesma cepa em camundongos C57BL/6 selvagens e transgênicos *knock-out* para o gene ativador de recombinação 1 (Rag-1), e em camundongos BALB/c e SCID. Tanto os animais Rag-1 *knock-out* quanto dos SCID não desenvolvem linfócitos B e T funcionais. Estes animais desenvolveram doença grave letal e alta freqüência de hemorragia pulmonar. Estas observações sugerem, portanto, que a auto-

imunidade/imunidade adaptativa provavelmente não tem um papel importante na patogênese da hemorragia pulmonar na leptospirose experimental.

5. Discussão

Neste estudo confirmamos relatos anteriores, que utilizam camundongos de outras linhagens (C3H e C3H/HeJ), de que a imunodeficiência resulta em alta suscetibilidade à infecção aguda, progredindo rapidamente para a morte (NALLY et al., 2005b, VIRIYAKOSOL et al., 2006). Além disso, a imunodeficiência resultou em altas cargas de leptospiras nos órgãos-alvo, observado no momento da necropsia, de acordo com o relato anterior em camundongos C3H/SCID (NALLY et al., 2005b). Observamos patologia típica nos órgãos alvo associado a leptospirose incluindo lesão tubular aguda e destrabeculação dos hepatócitos, semelhante à patologia descrita em seres humanos e em modelos de leptospirose letal, tais como hamsters (AREAN, 1962; SILVA et al., 2008).

Não existem dados suficientes para atribuir a patogênese da leptospirose relacionada com hemorragia pulmonar a um único mecanismo. Além disso, é razoável assumir que possa ser um resultado de um quadro multifatorial de efeitos tóxicos diretos da exposição a leptospiras, os efeitos da inflamação sistêmica na parede alveolar, distúrbios hemostáticos e uremia (MEDEIROS et al., 2010). Nally et al descreveram depósitos lineares de anticorpos e complemento no modelo de cobaia, sugerindo um papel potencial de auto-anticorpos contra antígenos *in situ* (da parede alveolar) na patogênese da leptospirose associada à hemorragia pulmonar (NALLY et al., 2004). Este mecanismo proposto aproximaria a patogênese da SPHS com a síndrome de Goodpasture, onde auto-anticorpos contra o colágeno tipo IV são dirigidos contra a membrana basal glomerular (GBM) reagindo de forma cruzada com a matriz do septo alveolar, causando hemorragia alveolar maciça. No entanto, a avaliação de anticorpos anti-GBM do soro de pacientes de Salvador com leptospirose não encontraram associação entre anticorpos anti-GBM e doença pulmonar. Não houve diferença nos níveis séricos de anticorpos anti-GMB entre os pacientes e os grupos controle para ambos os soros de fase aguda ou convalescente (CRODA, 2008).

Além disso, Craig et al não encontraram nenhuma evidência de anticorpos anti-GBM em 40 pacientes com leptospirose (CRAIG et al., 2009). No presente estudo, os camundongos que eram incapazes de produzir linfócitos B e T funcionais desenvolveram hemorragia alveolar grave. Este resultado sugere que a auto-imunidade não é um mecamismo importante para a hemorragia pulmonar na leptospirose experimental, pelo menos no modelo murino e/ou em infecções com *Leptospira interrogans* sorovar Copenhageni.

A tabela 3 do artigo apresentado mostra que os *imprints* de tecidos revelam quantificações muito variáveis nos órgãos alvo de camundongos imunodeficientes, submetidos à eutanásia durante a fase aguda da doença. Nos rins, essa quantificação é muito maior do que aquela observada nas linhagens de camundongos selvagens, necropsiados no 28º dia de infecção. Tal comparação é difícil de ser interpretada porque essas diferenças podem ser explicadas tanto pelo estado imunológico quanto pela diferente fase da infecção. Chama atenção, no caso dos camundongos imunodeficientes, que uma alta contagem de leptospiras pode ser observada nos pulmões, sendo comparável aos valores encontrados no rim ou no fígado. Em geral, as descrições da leptospirose humana e experimental chamam atenção para uma quantidade pequena de leptospiras nos pulmões. Algumas observações, no entanto, são importantes. Primeiro, a detecção de leptospiras nos pulmões na maioria dos estudos em humanos e animais baseia-se em imunohistoquímica (SILVA et al., 2002; PEREIRA et al., 2005; SPICHLER et al., 2007) e, portanto, cortes de pulmão sempre terão uma quantidade menor de tecido a ser avaliado quando comparado com órgãos sólidos. A técnica de *imprint* do pulmão cria um fluido que cobre uma área maior da lâmina e, por isso, pode permitir uma contagem de organismos que seria subestimada em cortes de tecidos submetidos à imunofluorescência ou imunohistoquímica. Neste caso, a melhor maneira de avaliar essa questão seria a quantificação não por um campo histológico e sim por grama de tecido. A quantificação por PCR em tempo real (expressa em leptospiras por grama de tecido ou em grama de DNA extraído) resolveria a questão. No entanto, poucos estudos usam essa metodologia especialmente para quantificação de leptospiras no pulmão. Em estudo de pacientes com leptospirose grave no Peru, um caso de hemorragia pulmonar teve a carga de leptospira quantificada no pulmão levando a um resultado de 10^6 leptospiras/g, um valor igual ou maior ao observado

no rim, soro e urina do mesmo paciente e de outros pacientes (SEGURA et al*.,* 2005). Da mesma forma, um estudo com base em PCR quantitativo em hamsters demonstrou que durante o processo de disseminação, as leptospiras atingem picos de contagem no pulmão que variam entre 10^6 e 10^7 leptospiras/g, não diferindo da quantificação observada em outros órgãos alvo tais como rim ou fígado (TRUCCOLO et al*.,* 2002). Em segundo lugar, a alta contagem de leptospiras nos pulmões implicaria um mecanismo de hemorragia pulmonar similar ao que ocorre na sepse grave resultando em lesão alveolar difusa (o substrato morfológico da síndrome da angustia respiratória do adulto, SARA). É interessante notar que na leptospirose são freqüentes tantos os achados compatíveis com lesão alveolar difusa / SARA (MEDEIROS et al*.,* 2010) quanto achados característicos da sepse bacteriana tais como a associação de marcadores pró-inflamatórios com a gravidade da doença (TAJIKI e SALOMÃO, 1996; WAGENAAR et al*.,* 2009), e o mesmo perfil de alterações hemodinâmicas (SIRIWANIJ et al*.,* 2005).

A deficiência genética da iNOS em camundongos não teve nenhum efeito sobre a sua sobrevivência ou o desenvolvimento de anticorpos aglutinantes ou anticorpos específicos IgG anti-*Leptospira*. Em teoria, a produção prejudicada de NO durante a resposta imune pode estar relacionada com maiores cargas de leptospiras em tecidos devido a sabida interação entre o NO e radicais livres do oxigênio que cooperam na atividade microbicida em fagócitos. No entanto, a maior carga de *Leptospira* observada não foi reproduzida em experimentos independentes. A única diferença significativa observada entre camundongos iNOS-KO e do tipo selvagem C57BL/6 é que os animais transgênicos desenvolvem nefrite intersticial a uma taxa menor e em graus menos graves, que o controle selvagem. Este resultado está de acordo com a hipótese de que a expressão de marcadores pró-inflamatórios pelas células tubulares renais *in vitro* após a exposição a produtos da *Leptospira* pode estar relacionado com o desenvolvimento de nefrite intersticial *in vivo* (YANG et al., 2006; YANG et al., 2002; YANG et al., 2000).

6. Conclusão

A ausência de linfócitos B e T funcinais não impedem a ocorrência de hemorragia pulmonar. Essas informações fornecem fortes argumentos de que a hemorragia pulmonar na leptospirose não está relacionada apenas a mecanismos auto-imunes nos modelos murinos estudados. A ausência de um gene funcional iNOS no modelo murino teve um efeito mínimo sobre o resultado da leptospirose, exceto para uma suscetibilidade significativamente reduzida para o desenvolvimento de nefrite intersticial ou de suas formas mais graves, pelo menos no modelo murino e/ou em infecções com *Leptospira interrogans* sorovar Copenhageni cepa Cop.

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8. ANEXO I

Outras publicações na área de leptospirose experimental durante o período do mestrado.

SANTOS, C.S., MACEDO, J.O., BANDEIRA, M., CHAGAS-JUNIOR, A.D., MCBRIDE, A.J., MCBRIDE, F.W., REIS, M.G., ATHANAZIO, D.A. Different outcomes of experimental leptospiral infection in mouse strains with distinct genotypes. J Med Microbiol. Sep;59 (Pt 9):1101-6. 2010.

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O modelo murino oferece múltiplas ferramentas para estudos imunológicos e genéticos. Alguns estudos avaliando a infecção por leptospiras patogênicas em camundongos mutantes ou transgênicos veem sendo relatados, no entanto, o uso mais amplo desses modelos experimentais é limitado por nossa pouca compreensão sobre o desfecho da infecção por leptospiras patogênicas em linhagens murinas selvagens. Neste estudo, relatamos o desfecho da infecção por *Leptospira interrogans* sorovar Copenhageni cepa Cop em quatro linhagens murinas selvagens amplamente usadas em pesquisa básica: A, CBA, BALB/c e C57BL/6. Os desfechos avaliados foram sobrevida, presença de lesões renais, carga de leptospiras nos rins, títulos de anticorpos aglutinantes séricos e níveis séricos de IgG específica anti-*Leptospira*. Como esperado, nenhuma linhagem mostrou-se susceptível à doença aguda letal. As linhagens A e C57BL/6 apresentaram maior carga renal de leptospiras, enquanto os camundongos CBA e C57BL/6 apresentaram lesões renais mais graves. Por outro lado, camundongos BALB/c demonstraram notável resistência inclusive à leptospirose subclínica.

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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 intra-alveolar 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

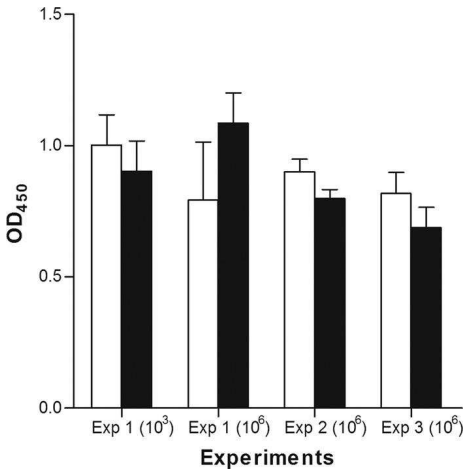

FIG. 1. ELISA analysis of serum anti-leptospiral IgG levels in infected mice 28 days postinfection. The graph compares antibody levels in C57BL/6 wild-type mice (solid bars) and inducible nitric oxide synthase knockout mice (open bars). The error bars represent the standard error of the mean for each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

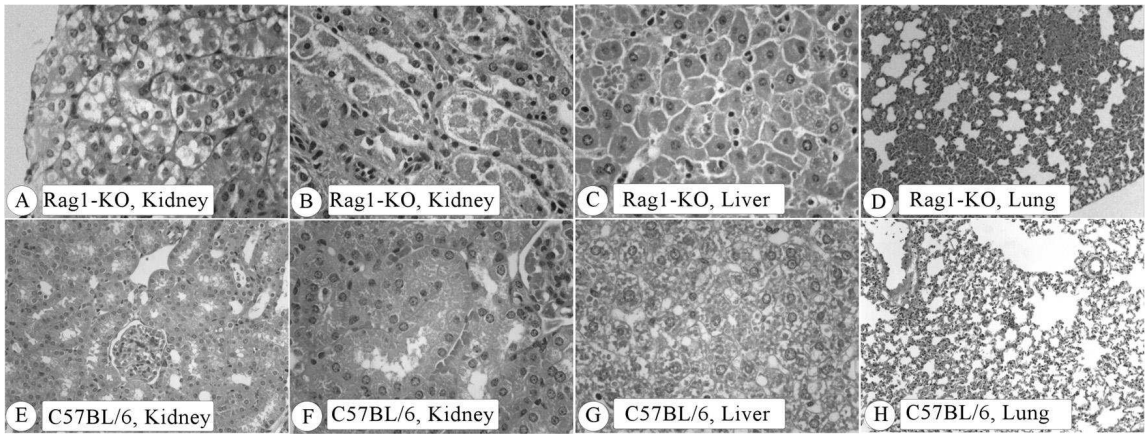

FIG. 2. Typical lesions of leptospirosis in a recombination activating gene 1 knockout C57BL/6 mouse that died 9 days after infection with strain Cop at a 10^6 inoculum. All tissue samples are stained with hematoxylin and eosin. (A) Marked cell swelling of epithelial cells of proximal tubules (×400). (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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Different outcomes of experimental leptospiral infection in mouse strains with distinct genotypes

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The mouse disease model has the advantage of a broad array of immunological and genetic tools available for basic research. Some studies on transgenic and/or mutant mouse strains as models for experimental leptospirosis have been reported; however, the wider use of such models is hampered by a poor understanding of the outcome of experimental leptospiral infection among the different mouse strains available. Here, the outcome of infection by a virulent strain of *Leptospira interrogans* serogroup Icterohaemorrhagiae strain Cop was studied in four commonly used wild-type mouse strains: A, CBA, BALB/c and C57BL/6. The end points evaluated in this study were survival, presence of kidney lesions, leptospiral load in kidney samples, microscopic agglutination test titre and anti-leptospiral IgG antibody levels. As expected, none of the mouse strains were susceptible to lethal leptospirosis. However, these strains developed specific pathologies associated with sublethal leptospirosis. The A and C57BL/6 strains exhibited a high leptospiral load in kidney samples and the CBA and C57BL/6 strains developed severe inflammatory lesions, whilst the BALB/c strain proved to be the most resistant to subclinical leptospirosis.

INTRODUCTION

Leptospirosis is a widespread zoonosis with a broad clinical spectrum including fatal outcomes due to acute renal failure and pulmonary haemorrhage. Pathogenic leptospires are carried by diverse mammalian reservoirs, and peridomiciliary rodents are the most important source of infection in urban settings (McBride *et al.*, 2005). Despite major efforts to develop new vaccine strategies with a long-lasting effect and cross-protection against different serovars, our current knowledge of the immune determinants involved in host protection and pathogenesis remains limited (McBride *et al.*, 2005).

Diverse experimental animal models are used in leptospirosis research. Guinea pigs and hamsters are the most suitable laboratory rodents for reproducing acute lethal infection (Nally *et al.*, 2004; Silva *et al.*, 2008; Spichler *et al.*, 2007), whereas rats represent a prototype of resistance to acute disease and a potential model for persistent infection (Athanazio *et al.*, 2008b; Nally *et al.*, 2005b). Mice usually develop a persistent asymptomatic carrier state and are therefore recognized as resistant hosts, although this has not been studied in detail in a broad range of mouse strains. Swiss mice may succumb or develop subclinical renal colonization depending on their age and on inoculum size (Faine, 1962). Mouse models have the advantage that a broad array of immunological and genetic tools is available for basic research. Several studies on transgenic and/or mutant mouse strains have been carried out (Athanazio *et al.*, 2008a; Nally *et al.*, 2005a; Viriyakosol *et al.*, 2006); however, the wider use of such tools is hampered by a lack of understanding of the outcome of experimental leptospiral infection among the different mouse strains. For instance, the lack of effect of a single gene knockout (such as interleukin-4 in BALB/c mice) could be due potentially to an intrinsic resistance to the disease in wild-type mice (Athanazio *et al.*, 2008a). In addition, the induction of higher expression levels of pro-inflammatory mediators in murine renal tubular cells *in vitro* implicates leptospire-derived products in interstitial nephritis (Yang *et al.*, 2000, 2002, 2006). This view ignores the fact that interstitial nephritis is a late feature of human leptospirosis, probably reflecting secondary lesions following acute tubular damage (Arean, 1962). Also, it is not known to what extent mice develop interstitial nephritis after experimental leptospiral infection. In a previous report, we observed mild inflammatory changes in convalescent C57BL/6 mice, whereas the BALB/c strain failed to exhibit any subclinical pathology (Athanazio *et al.*, 2008a).

Abbreviations: MAT, microscopic agglutination test; p.i., post-infection.

The aim of this study was to clarify the outcome of experimental leptospiral infection among four commonly used laboratory mouse strains. We tested low and high infective doses of a highly virulent *Leptospira interrogans* serogroup Icterohaemorrhagiae isolate and investigated five end points of infection: survival, renal pathology, renal colonization in the convalescent phase by immunofluorescence of imprint samples (Chagas-Junior *et al.*, 2009), immune responses by microscopic agglutination test (MAT) and an in-house IgG ELISA.

METHODS

***Leptospira* strain and culture conditions.** Leptospires were cultivated in liquid Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (Difco Laboratories) at 29 °C and counted in a Petroff–Hausser counting chamber (Fisher Scientific). An isolate from Brazil, *L. interrogans* serogroup Icterohaemorrhagiae strain Cop, was used in all assays. 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. In previous experiments, the virulence of this strain at this passage was evaluated in hamsters and the 50 % lethal dose was calculated to be ~164 leptospires.

Experimental infection of mouse models of leptospirosis. Four groups of wild-type mice strains (A, CBA, BALB/c and C57BL/6) were infected intraperitoneally with a low (10^3) or a high (10^6) infective dose of *L. interrogans* serogroup Icterohaemorrhagiae strain Cop. Control groups were inoculated with 1 ml sterile EMJH medium. All animals were females; the A, BALB/c and C57BL/6 strains were 4–5 weeks old, whilst the CBA strain mice were 6–7 weeks old. The A strain used in this study refers to the A/J Unib substrain derived from the A/J strain and maintained at Cemib (Multidisciplinary Centre for Biological Research), University of Campinas, Brazil. The same institution was the source of the CBA and BALB/c mice used in this study, whilst the C57BL/6 mice were supplied by CAECAL (Laboratory Animal Breeding Centre), Oswaldo Cruz Foundation (Fiocruz). All four strains were originally purchased from The Jackson Laboratory.

Light microscopy. Euthanasia was performed on animals at 28 days post-infection (p.i.) and necropsies were carried out immediately following euthanasia. One kidney was fixed in 4 % formalin, embedded in paraffin and sections of 4–5 μm were used for conventional histology. A semi-quantitative estimation of interstitial nephritis was used with the following criteria: grade +, infiltrates rich in macrophages and lymphocytes restricted to periarterial areas; grade ++, infiltrates extending to other renal parenchymal zones with one to two lesions per field of view at ×400 magnification; and grade +++, the same lesion was detected in more than two areas per field of view at ×400 magnification (Athanazio *et al.*, 2008a). Grade ++ and +++ lesions were considered to be severe for analysis purposes.

Immunofluorescence of imprint samples. Imprints were obtained by direct pressure of the cut surface of the kidney sample onto a poly-L-lysine-coated glass slide as described previously (Chagas-Junior *et al.*, 2009). Briefly, the imprint slides were dried at room temperature, fixed in acetone for 3 min and the smear regions on the slides were demarcated with a hydrophobic barrier pen. Imprint slides were incubated for 60 min with a primary rabbit polyclonal anti-leptospiral antibody at a dilution of 1 : 200. After three washes in PBS, the imprints were incubated with goat anti-rabbit IgG–FITC conjugate at a dilution of 1 : 500, washed three times in PBS and dried before visualization of stained organisms by fluorescence microscopy. Kidney samples from non-infected mice were used as negative controls. Counterstaining of nucleic acids or cell cytoplasm was achieved with 1 mg 4′,6-diamidino-2-phenylindole ml^{-1} (Sigma-Aldrich) at room temperature for 10 min or with 0.1 % Evans Blue (Sigma Aldrich), respectively. Leptospires were quantified in imprint samples as the mean number of leptospires in 10 fields of view at a magnification of ×400. Only intact spiral-shaped organisms were included in the calculation.

Serology assays. The MAT was performed as described previously (WHO, 2003) except that only the *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni reference strain M20 was used as the live antigen.

Cells from 7-day-old cultures of *L. interrogans* serogroup Icterohaemorrhagiae strain Cop were harvested by centrifugation (1500 ***g*** for 30 min at 4 °C) and washed three times by centrifugation in PBS. The cells were resuspended in 0.1 M Na_2CO_3 (pH 9.6), at a concentration of approximately 10^8 cells ml^{-1}. The cells were inactivated by heating at 56 °C for 20 min. The resulting material was stored at −20 °C and used as the antigen for ELISA. An in-house IgG ELISA was used to compare anti-*Leptospira* IgG levels among the various mouse strains used in this study. The optimal concentrations of the antigen, mouse sera and antibody conjugates were determined in a preliminary chequerboard analysis. The equivalent of ~10^7 inactivated whole leptospires (*L. interrogans* serogroup Icterohaemorrhagiae strain Cop) in 100 μl 0.1 M Na_2CO_3 (pH 9.6) was adsorbed onto the surface of each microtitre well at

Table 1. Presence of nephritis, leptospiral count and MAT titres in mice at 28 days p.i.

QD, Quartile deviation; ID, infective dose.

Mouse strain	% Nephritis (no./total)*			Leptospiral count (mean ± SD)		MAT titre (median ± QD)	
	Control	Low ID	High ID	Low ID	High ID	Low ID	High ID
A	0 (0/20)	66.7 (10/15)†	40.0 (8/20)†	20 ± 16	26 ± 21	800 ± 800	800 ± 1200
CBA	0 (0/20)	78.9 (15/19)†	60.0 (12/20)†	10 ± 11	10 ± 10	1200 ± 900	800 ± 300
BALB/c	0 (0/19)	0 (0/20)	0 (0/20)	5 ± 9	5 ± 7	400 ± 200	200 ± 300
C57BL/6	0 (0/20)	90.0 (27/30)†	92.0 (23/25)†	19 ± 27	15 ± 16	800 ± 600	800 ± 400

*Histopathological evidence for interstitial nephritis, grade + or ++ (see Table 2 for further details).
†$P<0.01$ compared with the uninfected control group.

Table 2. Level and severity of interstitial nephritis in infected mice at 28 days p.i.

Grade: +, mild lesions; ++, moderate lesions; +++, severe lesions.

Mouse strain	% Nephritis (no./total)								
	Uninfected controls			Low infective dose			High infective dose		
	+	++	+++	+	++	+++	+	++	+++
A	0 (0/20)	0 (0/20)	0 (0/20)	53.3 (8/15)	13.3 (2/15)	0 (0/15)	40.0 (8/20)	0 (0/20)	0 (0/20)
CBA	0 (0/20)	0 (0/20)	0 (0/20)	47.4 (9/19)	31.6 (6/19)	0 (0/19)	50.0 (10/20)	10.0 (2/20)	0 (0/20)
BALB/c	0 (0/19)	0 (0/19)	0 (0/19)	0 (0/20)	0 (0/20)	0 (0/20)	0 (0/20)	0 (0/20)	0 (0/20)
C57BL/6	0 (0/20)	0 (0/20)	0 (0/20)	40.0 (12/30)	50.0 (15/30)	0 (0/30)	32.0 (8/25)	60.0 (15/25)	0 (0/25)

37 °C for 1 h. The wells were washed five times with 0.05 % Tween 20 in PBS (PBS-T) and incubated for 1 h at 37 °C with 200 µl blocking solution (PBS-T plus 1 % BSA). Mouse serum diluted 1 : 200 in blocking solution was added and incubated for 1 h at 37 °C. After five washes with PBS-T, anti-mouse horseradish peroxidase-conjugated antibody (1 : 2000; Dako Cytomation) was added and incubated for 1 h at 37 °C. After five washes with PBS, 100 µl substrate solution (1 mg 3,3′,5,5′-tetramethylbenzidine dissolved in 1 ml DMSO, 9 ml 0.05 M phosphate citrate buffer, 2 µl 30 % H_2O_2) was added to each well. The colour reaction was allowed to proceed for 15 min and stopped with 25 µl 1 M H_2SO_4. The plate was read in a microplate reader (GENios; Tecan) at A_{450}.

Statistics. Statistical analyses were performed using the GraphPad Prism 4.03 software package. Categorical data were compared by Fisher's exact test and numerical data were compared using a non-parametric Mann–Whitney test (two groups) or a Kruskal–Wallis test (more than two groups).

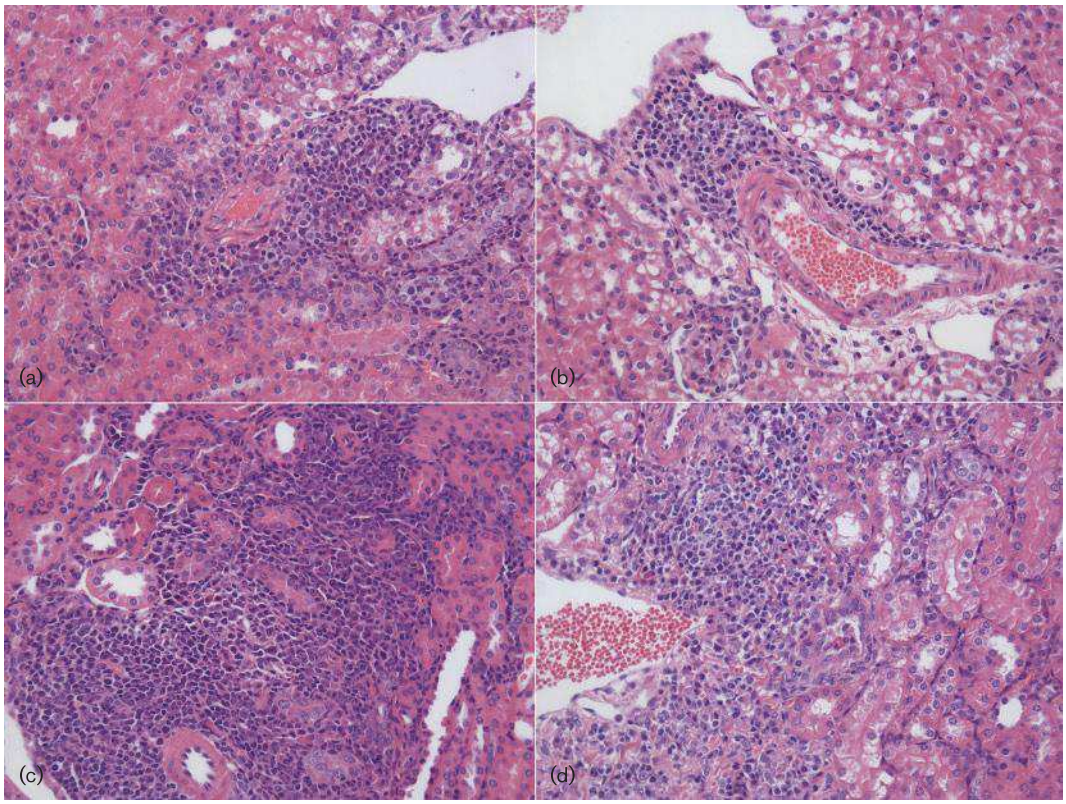

Fig. 1. Illustrative images of interstitial nephritis grading in infected mice. (a, b) Kidney sample from a C57BL/6 mouse infected with a low infective dose (10^3 leptospires) exhibiting nephritis grade +, characterized by infiltrates rich in macrophages and lymphocytes restricted to periarterial areas. (c, d) Kidney sample from a C57BL/6 mouse infected with a low infective dose exhibiting nephritis grade ++, characterized by inflammatory infiltrates extending to other renal parenchymal zones. Magnification, ×400.

RESULTS

None of the infected or control (uninfected) animals exhibited any clinical symptoms of leptospirosis; therefore, they were euthanized and necropsied at 28 days p.i. Kidney samples were examined microscopically for evidence of interstitial nephritis. None of the control animals, regardless of the mouse strain, exhibited lesions. Of note, none of the infected BALB/c mice, regardless of the size of the infective dose, exhibited inflammatory lesions. Table 1 summarizes the frequency of interstitial nephritis observed. The A, CBA and C57BL/6 strains presented with significantly more lesions than the controls ($P<0.01$) for both the lower and higher infective doses. However, the size of the infective dose in these strains had no significant effect on the frequency of interstitial nephritis observed. Detailed information on the severity of interstitial nephritis and the infective dose is presented in Table 2. Illustrative images of the interstitial nephritis grades + and ++ observed in this study are shown in Fig. 1.

The number of leptospires in kidney samples collected from the mouse strains at 28 days p.i. was determined by immunofluorescence-based detection in imprint samples (Table 1). No spirochaetes were detected in the uninfected controls. Strains A, CBA and C57BL/6 exhibited significantly higher ($P<0.001$) loads of leptospires compared with the BALB/c infected groups. This difference was observed for both infective doses used in this study. Significantly higher numbers of leptospires were detected in the A strain at the higher infective dose. Interestingly, the inverse was observed in the C57BL/6 strain, where a significantly higher leptospire count was seen in the group that received the lower infective dose. Representative images of the immunofluorescence-based detection of leptospires in imprint samples from mouse kidney samples are show in Fig. 2.

MAT quantification of agglutination antibodies against serovar Copenhageni is presented in Table 1. This revealed lower titres (1 : 400 and 1 : 200) in the BALB/c groups, regardless of the size of the infective dose. In the other mouse strains, the median MAT titres were significantly higher and ranged from 1 : 800 to 1 : 1200. However, there was considerable variation of the MAT titres within the individual groups, as reflected in the quartile deviations presented in Table 1. The specific anti-leptospiral IgG levels in the infected mice strains were determined by ELISA (Fig. 3). The IgG levels were significantly higher in the infected animals than in the control groups. In the A and BALB/c strains, the higher infective dose stimulated significantly higher IgG levels ($P<0.05$) compared with the lower infective dose. When the IgG responses between strains were compared, the C57BL/6 mice, at the lower infective dose, exhibited significantly higher IgG levels ($P<0.01$), and the BALB/c mice, at the higher infective dose, had significantly higher IgG levels ($P<0.05$) (Fig. 3).

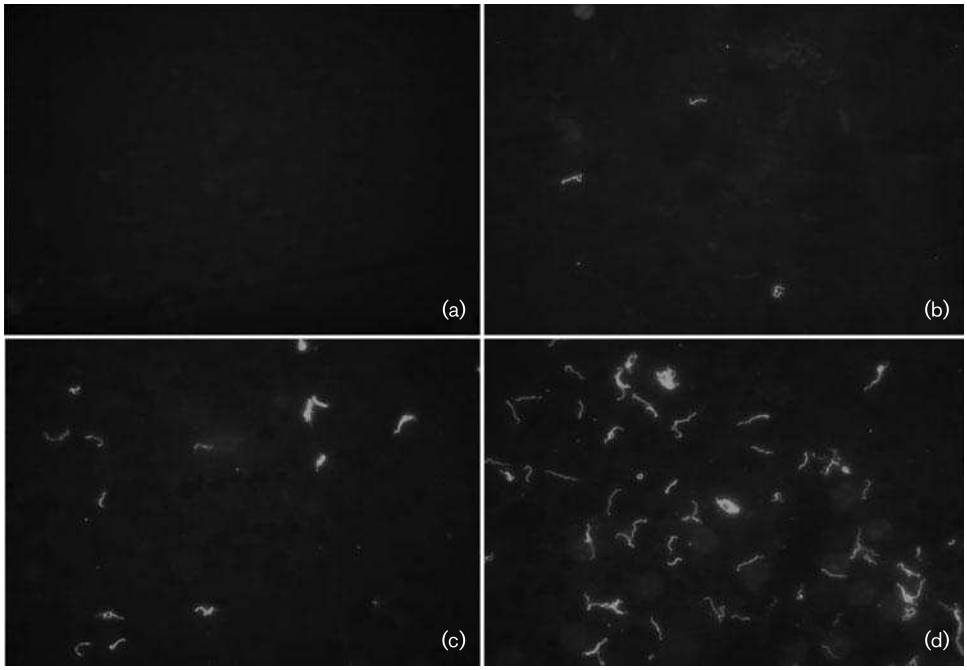

Fig. 2. Illustrative images of immunofluorescence-based detection of leptospires in imprint samples from representative mouse kidneys. (a) An A strain mouse inoculated with sterile EMJH medium (uninfected control). (b) BALB/c mouse infected with a low infective dose (10^3 leptospires): ~3 leptospires per ×400 field of view. (c) A CBA strain mouse infected with a low infective dose: ~12 leptospires per ×400 field of view. (d) An A strain mouse infected with a low infective dose: ~46 leptospires per ×400 field of view. Magnification, ×400.

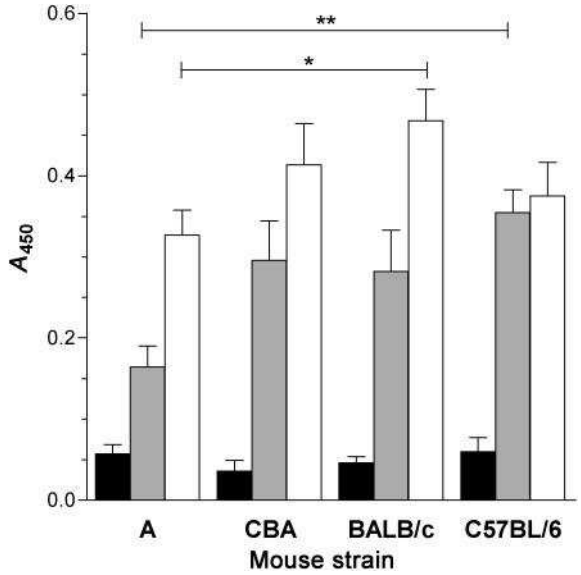

Fig. 3. Analysis of serum anti-leptospiral IgG levels in infected mice at 28 days p.i. An ELISA was used to determine IgG levels in sera collected from a control (uninfected) group (filled bars), a low infective dose (10^3 leptospires) group (shaded bars) and a high infective dose (10^6 leptospires) group of mice (open bars). Results are shown as means ± SEM for each group. The statistical significance between groups was calculated using a Mann–Whitney test: *, $P<0.05$; **, $P<0.01$.

DISCUSSION

Based on our observations in this study, the general concept that mice are relatively resistant to severe leptospirosis appears to hold true for many of the widely used laboratory strains. As expected, none of the mouse strains proved to be a model of lethal leptospirosis. However, our results indicated that certain strains could be useful for studies of experimental leptospirosis. The A and C57BL/6 mouse strains exhibited significantly higher leptospiral loads in kidney samples, and this may prove advantageous when studying *Leptospira* species recovered from renal tubules, as large numbers of leptospires are required in such studies. The A, CBA and C57BL/6 strains tended to develop more inflammatory lesions, suggesting that they may be the most suitable strains for studies on interstitial nephritis. Furthermore, the C57BL/6 strain displayed significantly more kidney lesions at the high infective dose than any of the other strains (Tables 1 and 2). This is in agreement with a previous study, where we reported higher susceptibility in the C57BL/6 strain compared with the BALB/c strain (Athanazio *et al.*, 2008a).

Our data on the immune responses support only preliminary interpretations; however, it is interesting to note that the A strain displayed one of the highest leptospiral loads observed in the kidneys and significantly lower levels of specific anti-*Leptospira* IgG. In contrast, BALB/c mice exhibited both significantly fewer kidney lesions (zero) and a lower leptospiral load (independent of the infective dose), together with high levels of IgG, significantly so when compared with the A strain at the higher infective dose. Furthermore, the IgG response in both of these strains was dose dependent (Fig. 3). In addition, significantly higher levels of IgG were observed in the C57BL/6 strain compared with the A strain at the lower infective dose. Leptospiral load in the kidneys of both of these strains was comparable and, although the frequency of interstitial nephritis was higher in the C57BL/6 strain, it was not significant at the lower infective dose (Table 1). However, it was significant when only severe nephritis (grade ++) was evaluated at both the low and high infective dose when comparing the A and C57BL/6 strains (Table 2). It is possible that the lower MAT titres observed in the BALB/c strain may be due to the rapid clearance of leptospires during the initial stages of infection. However, the IgG levels observed in the BALB/c groups seem to contradict this conclusion.

Experimental data on leptospiral infection in mice are sporadic and fragmented. Passive immunization experiments and transfer of splenic B cell subsets in cyclophosphamide-treated BALB/c mice have suggested that humoral immunity is the key issue in natural resistance to disease. Athymic nude mice showed no difference in susceptibility when compared with wild-type animals (Adler & Faine, 1977). Transgenic and mutant murine models have been used to study leptospirosis, yet most studies have explored only the role of innate immunity. Toll-like receptor (TLR)-4 defective C3H/HeJ mice have been used to evaluate recombinant protein vaccine candidates (Koizumi & Watanabe, 2004). Double-knockout (TLR2 and TLR4) C57BL/6 mice were also found to be highly susceptible to lethal disease (Chassin *et al.*, 2009). TLR2-defective C57BL/6 mice were found to be resistant to the toxic effects of leptospiral LPS, but the role of TLR2 in host innate protection was not evaluated (Werts *et al.*, 2001). Furthermore, severe combined immunodeficiency C3H and C3H/HeJ mice are highly susceptible to lethal infection (Nally *et al.*, 2005a; Viriyakosol *et al.*, 2006). Recently, the BALB/c and C3H/HeJ strains were used as models of resistance and susceptibility, respectively (da Silva *et al.*, 2009). Similar results for wild-type mice have been reported for leishmaniasis, where a C57BL/6 strain was resistant whilst BALB/c mice were susceptible to infection (Barral-Netto *et al.*, 1987).

Our results indicate recommended strains for future research in experimental leptospirosis. Among those tested, the A strain may be the strain of choice for studies aiming to recover large numbers of leptospires from colonized kidneys. Mouse strains CBA and C57BL/6 most frequently developed inflammatory lesions and would be the most suitable for studies on leptospirosis-associated interstitial nephritis. BALB/c mice are the strain of choice for studying mechanisms that involve innate immunity and/or rapidly adaptive immune responses. Additionally, the availability of a wide range of genetically manipulated mouse strains

opens a wide range of possibilities for analysing leptospiral pathogenesis.

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