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Short communication

Leptospira interrogans requires heme oxygenase for disease pathogenesis

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Abstract

We recently characterised the *Leptospira interrogans* heme oxygenase (*hemO*) gene and showed that HemO was required for growth with hemoglobin as the sole iron source. Here we investigated the role of HemO in pathogenesis. Hamsters inoculated with the *hemO* mutant showed 83% survival, compared with 33% for a control mutant (intergenic transposon insertion). Lung pathology was consistent with survival data, showing that HemO contributes significantly to pathogenesis and heme is a major *in vivo* iron source for *L. interrogans*. This is only the second defined, attenuated mutant in pathogenic *Leptospira* and the first to define function of the mutated gene.

Keywords: *Leptospira interrogans*; Heme oxygenase; Hemoglobin; Pathogenesis

1. Introduction

Leptospirosis is a widespread zoonosis contracted through either direct contact, or indirect contact via contaminated water or soil, with urine from infected carrier animals. *Leptospira interrogans* is the most common cause of severe leptospirosis worldwide. In humans, disease syndromes range from a mild, flu-like illness through to severe pulmonary hemorrhage and multiple organ failure [1].

In the mammalian host, the availability of iron is highly restricted, so pathogens have evolved complex iron acquisition strategies [2]. One strategy is to obtain iron from the most abundant *in vivo* source, heme. Several lines of evidence suggest that *L. interrogans* utilises heme *in vivo*. *L. interrogans* is hemolytic, with multiple sphingomyelinases hypothesised to liberate hemoglobin from erythrocytes. Leptospires are chemotactic towards hemoglobin [3], and the sequenced genomes of *L. interrogans* possess multiple genes with putative involvement in heme acquisition, such as those encoding TonB-dependent receptors and ABC transport genes [4]. Significantly, *L. interrogans* can grow with heme or hemoglobin as the sole iron source [5,6].

Research into mechanisms of pathogenesis in leptospirosis has been severely hampered by the lack of genetic techniques for pathogenic *Leptospira*. Transposon mutagenesis has only recently been achieved [5], and has so far only led to the identification of only one virulence associated protein [6]. Using transposon mutagenesis we recently described a defined mutation in the heme oxygenase gene, *hemO*, of *L. interrogans* [7]. Bacterial heme oxygenases are cytoplasmic enzymes that degrade the heme tetrapyrrole ring, releasing ferrous iron for use by the cell [2]. The *L. interrogans* HemO protein has high identity to other heme oxygenases, and was characterised as a functional enzyme. Growth assays demonstrated that it was required for growth when hemoglobin was the sole iron source

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[7]. In this study, we show that heme oxygenase contributes significantly to disease causation in the hamster model of infection, confirming the importance of heme as an *in vivo* source of iron. This is only the second description of a defined, attenuated mutant in *L. interrogans*, and the first in which the function of the mutated gene is defined. This study is also the first description of a bacterial heme oxygenase contributing significantly to virulence.

2. Materials and methods

2.1. Bacterial strains

L. interrogans serovar Manilae *hemO* mutant M484 was constructed by transposon mutagenesis using the *Himar1*-derived transposon Tn*SC189* [7]. To control for attenuation that may result from the *in vitro* culture necessary in the transformation process, the control strain M511 was selected from the same batch of mutants as M484, but has an intergenic transposon insertion between 16S rRNA gene and LA2443. Complementation of strain M484 was not possible because there are no replicating vectors for use in *L. interrogans*.

2.2. Infection model in hamsters

In three separate experiments groups of 8 5-week old hamsters of either sex weighing 45–60 g were injected intraperitoneally with 10^3 leptospires in 100 μL of EMJH medium (Becton Dickinson, New Jersey, USA). Control animals (group of 4) received 100 μL EMJH medium or 10^3 wild type Manilae (groups of 8 and 4). A dose of 10^3 wild type Manilae causes 100% lethality (Table 1) in 6–8 days. Hamsters were monitored for 14 days and moribund animals were killed in accordance with animal ethics requirements. Kidneys were removed from animals for culture by aseptically removing the capsule and forcing the kidney through a 10 mL syringe into 5 mL of EMJH. The solution was allowed to settle for 5 min, then 50 μL were transferred to 5 mL fresh EMJH for culture. Urine was removed from the bladder by needle aspiration and one drop added to 5 mL EMJH for culture. Statistical analysis was conducted using Fisher's exact test.

For histopathological examination, tissue was fixed in 10% neutral-buffered formalin, dehydrated by immersion in increasing concentrations of ethanol, then xylene, before embedding in paraffin. Sections were cut and stained with hematoxylin and eosin.

3. Results

3.1. The heme oxygenase mutant is attenuated in hamsters

To determine whether heme oxygenase is required for disease pathogenesis, hamsters were inoculated with the heme oxygenase mutant (M484) or the control mutant M511 over three experiments. A negative control group was inoculated with 100 μL of medium.

In the group inoculated with M484 there was 83% (20/24) survival overall (Table 1), showing a highly significant difference from the control mutant M511 and wild type parent Manilae ($p = 0.001$ and 10^{-6}, respectively). Macroscopic examination of tissues showed that the animals which succumbed to infection had lung hemorrhage indicative of leptospirosis [1]. Surviving hamsters appeared healthy, though 75% of surviving animals showed evidence of minor pulmonary hemorrhage. Leptospires were isolated from the kidneys or urine of 85% of surviving animals. PCR analysis confirmed that leptospires recovered from kidney cultures retained the transposon insertion in *hemO* and had not reverted to wild type.

From the group inoculated with the M511 (intergenic mutant control) 33% (8/24) animals survived. The hamsters that succumbed to infection showed mild to severe pulmonary hemorrhage, while the survivors all showed pulmonary hemorrhage ranging from mild to severe pathology. All of the surviving hamsters and animals checked from the group that died were culture positive for leptospires. Control hamsters injected with EMJH remained normal throughout.

Histopathological examination was performed on hamsters infected with M484 (Fig. 1). The minor lung hemorrhages observed in some hamsters were confirmed by histopathology (Fig. 1a), while the remainder was normal (Fig. 1b). Kidney culture-positive hamsters showed minimal focal tubular necrosis (Fig. 1d), while hamsters that were kidney culture-negative had no renal pathology (Fig. 1e). The liver appearance was normal. These observations contrast with the pathology observed in wild type infections which showed typical severe lung hemorrhage, hepatic necrosis and acute renal tubular necrosis (data not shown).

Table 1
Assessment of virulence in the hamster model of infection.

Inoculum[a]	Hamster survival[b]				Culture positive[c]	
	Expt. 1	Expt. 2	Expt. 3	Total (%)	Survived (%)	Died (%)
M484	7/8	5/8	8/8	20/24 (83)[d]	17/20 (85)	3/3 (100)
M511	1/8	4/8	3/8	8/24 (33)	8/8 (100)	12/12 (100)
Manilae wild type	0/8	–	0/4	0/12 (0)	–	4/4 (100)
EMJH control	4/4	–	–	4/4 (100)	–	–

[a] Hamsters were inoculated with 10^3 leptospires in 100 μL EMJH.
[b] Animals were monitored for a period of 14 days.
[c] Animals were tested for *L. interrogans* colonisation by kidney or urine culture. Not all animals from each group were tested.
[d] $p = 0.001$ compared to M511 and 10^{-6} compared to wild type.

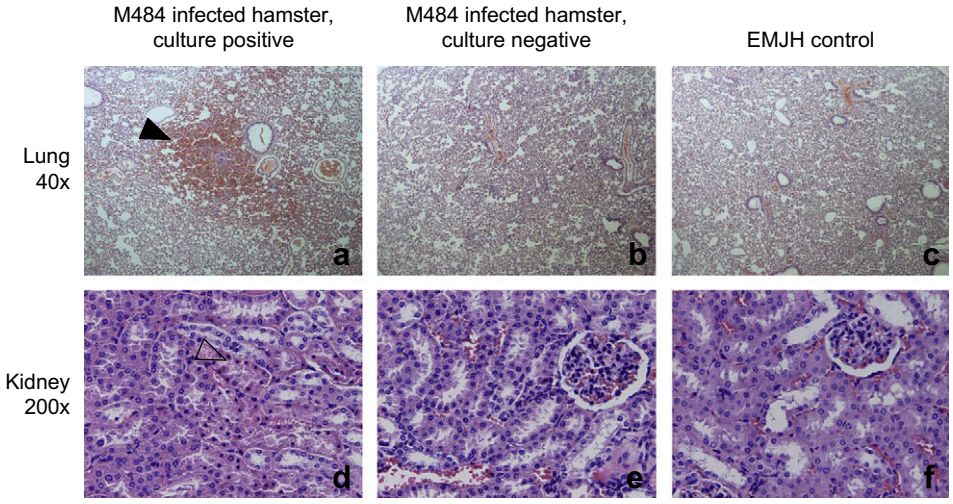

Fig. 1. Histopathological examination of hematoxylin and eosin stained tissues from hamsters. Top row: lung at 40× magnification; bottom row: kidney at 200× magnification. The solid arrow head indicates region of pulmonary hemorrhage, while the open arrow head indicates region of minimal ischemic tubular necrosis.

4. Discussion

Heme in the form of hemoglobin is the most abundant source of iron during infection, accounting for up to 75% of total iron in the host [8]. Analysis of the genome sequence of *L. interrogans* reveals the presence of genes encoding heme acquisition proteins, and is therefore consistent with the notion that *Leptospira* utilises heme as a major iron source during infection.

In this study, 83% of hamsters inoculated with the *hemO* mutant survived infection, showed little disease pathology and 15% were negative for renal carriage of leptospires at necropsy. Our results thus show that heme degradation by HemO contributes significantly to acute disease caused by *L. interrogans*, but is not essential. The result also suggests that heme is the main source of iron for *L. interrogans in vivo*. Disease was noted in some animals infected with the *hemO* mutant (four deaths, minor lung pathology in 75% of survivors), suggesting that leptospires may be utilising less abundant iron sources, though this is unlikely to involve siderophores [4]. Alternatively, *L. interrogans* may have another, less efficient, means of acquiring iron from hemoglobin; when hemoglobin was provided as the source of iron, the heme oxygenase mutant was significantly impaired in use of hemoglobin but still grew slowly in its presence [7].

There was an unexpected difference in the survival of hamsters infected with mutant M511 (33% survival) and wild type (0% survival; $p = 0.033$). Mutant construction of necessity involves a degree of *in vitro* passage. The slight attenuation observed was most likely a result of that *in vitro* culture.

It is interesting to note that the heme oxygenase gene is also present in the saprophytic species of *Leptospira biflexa*, presumably to control heme levels resulting from the endogenous heme biosynthesis locus [9].

While it has been shown that mutations affecting heme uptake can affect virulence [10], there has been little research into the direct role of heme oxygenase genes. Skaar et al. found that the heme oxygenase IsdG of *Bacillus anthracis* was not essential for virulence in the A/J mouse model of infection [11], while other investigators of bacterial heme oxygenases have not directly assessed their importance in virulence [12–16]. Therefore this is the first study finding that a heme oxygenase contributes significantly to virulence and is only the second description of a defined mutant in *L. interrogans* that results in attenuated virulence.

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