Establishment of a new murine model of liver abscess induced by *Fusobacterium necrophorum* injected into the caudal vein

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Anaerobic bacterial infection is often accompanied by abscess formation; however, few *in vivo* studies have been published with descriptive data specifically evaluating antimicrobial activity in the presence of abscesses. The aim of this study was to establish a murine model of anaerobic infection with abscess formation and to verify the utility of this model for evaluating the *in vivo* efficacy of an antimicrobial agent. A clinical isolate of *Fusobacterium necrophorum* was inoculated into the caudal vein of immunocompetent BALB/c mice at 10⁸ c.f.u. per mouse. Changes in body weight, bacterial load and histopathology of key organs were evaluated. After inoculation, bacterial counts in the liver increased from 10⁴ to 10⁸ c.f.u. after 1–3 days, and liver abscess formation was observed on the day following infection. Abscess formation and bacterial growth were not observed in other organs. In this model, 3 days of treatment with 5 mg metronidazole kg⁻¹ eradicated *F. necrophorum* in the liver; however, a reduction in bacterial load was not observed with 0.05 mg metronidazole kg⁻¹. In this study, we established a novel murine model of *F. necrophorum* liver abscess via haematogenous infection that may be useful for investigating *in vivo* antimicrobial activity against anaerobic abscesses and understanding the pathogenesis of *F. necrophorum* infection.

INTRODUCTION

Anaerobic bacteria cause several endogenous infections in humans that are associated with high mortality if untreated (Wilson & Limaye, 2004; Papaparaskevas *et al.*, 2011). Because anaerobes comprise a significant part of the normal flora of the human body, the identification of infectious organisms from clinical specimens and susceptibility testing of isolates is laborious and time consuming (Papaparaskevas *et al.*, 2011). Moreover, anaerobic infections often occur with abscesses, which impair the activity of antimicrobial agents and require surgical debridement as standard treatment (Giamarellou, 2000; Brook, 2012).

Although several reports have focused on the anti-anaerobic activities of newly developed antibiotics *in vitro* (Ednie & Appelbaum, 2009; Tanaka *et al.*, 2009; Tran *et al.*, 2011), there are few suitable animal models for testing the action of antimicrobial agents against anaerobic infections that are accompanied by abscesses.

In this study, we developed a murine model of liver abscess from *Fusobacterium necrophorum* through injection of the bacteria into the caudal vein. This model may be useful for studying the pathological aspects of infection and evaluating new therapeutic approaches. We also examined the efficacy of metronidazole (MTZ), a commonly used anti-anaerobic drug, against this model in order to verify the therapeutic response of the model to antibiotics.
METHODS

Mice. Eight-week-old male BALB/c specific-pathogen-free mice were obtained from SLC Japan. All mouse experiments were performed according to the guidelines of the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine. The Animal Care Ethics Review Committee of our institution approved the experimental protocol.

Organism. A clinical isolate of *F. necrophorum* subsp. *funduliforme* (strain FNU-89), maintained as a stock culture in the Department of Laboratory Medicine, Nagasaki University Hospital, Japan, was used in this study. We identified the strain by PCR amplification and sequencing analysis of the 16S rRNA gene. The MIC of MTZ against the organism, determined by the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute guidelines, was <0.004 mg l⁻¹.

Intravenous infection procedure. The *F. necrophorum* strain was cultured on PV Brucella HK agar (Kyokuto Pharmaceutical Industrial) for 48 h under anaerobic conditions. The culture was then scraped and suspended in modified Gifu Anaerobic Medium broth (Nissui Pharmaceutical) and cultured in an anaerobic chamber for 18 h. The bacteria were then harvested by centrifugation (3000 r.p.m. for 10 min). The organism was resuspended in physiological saline; the final number of bacteria prepared was approximately 10⁹ c.f.u. ml⁻¹. Bacteria were then harvested by centrifugation (3000 r.p.m. for 10 min). The bacteria were then harvested by centrifugation (3000 r.p.m. for 10 min). The bacteria were then harvested by centrifugation (3000 r.p.m. for 10 min). The bacteria were then harvested by centrifugation (3000 r.p.m. for 10 min). The bacteria were then harvested by centrifugation (3000 r.p.m. for 10 min).

Bacteriological and histopathological examination. Each group of animals was killed by cervical dislocation at specific time intervals. After exsanguination, the blood was collected and the liver, kidneys, spleen and lungs were dissected and removed under aseptic conditions. The organs used for bacteriological analyses were homogenized with a Polytron homogenizer and quantitatively inoculated onto Anaero Columbia rabbit blood agar (Becton Dickinson) by serial dilution. The liver tissue used for histological examination was fixed in 10% buffered formalin and stained with haematoxylin and eosin.

Drug treatment. MTZ treatment was evaluated in the liver abscess model. MTZ was obtained from Nacalai Tesque, reconstituted with acetic acid and further diluted in sterile physiological saline to the final concentrations for administration. To confirm dose-dependent drug efficacy, we administered MTZ at 5 mg kg⁻¹ as a sufficient treating dose and 0.05 mg kg⁻¹ as an insufficient treating dose. MTZ was administered daily via intraperitoneal injection for 3 days, starting on day 1 (24 h) after inoculum. Placebo mice received sterile physiological saline with the same amount of acetic acid contained in MTZ formulation. During the experiment, the body weight of the 1.6 × 10⁸ c.f.u. group remained significantly lower than that of control mice (23.1 ± 1.1 vs. 25.7 ± 1.4 g per mouse; *P*<0.05 compared with control groups). The 1.6 × 10⁷ c.f.u. group did not exhibit any continuous body weight decrease. Thus, an inoculum containing 1.6 × 10⁸ c.f.u. per mouse was considered sufficient to cause liver abscess formation, and we conducted the remaining experiments using inoculum at 1.6 × 10⁸ c.f.u. per mouse. The change in the number of viable *F. necrophorum* in the liver in the days following infection is shown in Fig. 1. The mean bacterial count in the liver of infected mice significantly increased from day 1 to 3 after infection. The bacterial count remained around 10⁷–10⁸ c.f.u. per liver for 7 days and decreased after 14 days. In order to distinguish co-culture or contamination by other bacteria, we concurrently cultured *F. necrophorum* obtained from the liver on PV Brucella HK agar and blood agar plates. The blood agar culture was conducted under aerobic conditions with a CO₂ incubator. No co-culture or contamination was found on the culture plates, and we determined that all bacterial counts obtained in the experiments were attributable to *F. necrophorum*. The bacterial number and gross abscess formation on day 4 after infection in the liver, blood, lungs, spleen and kidneys are described in Table 1. Gross abscess lesions could be detected only in the liver, and the mean bacterial load was significantly higher in the liver than in the other organs (*P*<0.01 for all). There was preferential abscess formation in the liver throughout the observation period.

Fig. 1. Bacterial load in the livers of mice infected with *F. necrophorum*. Each open circle represents the value for a mouse. Bacterial load in the liver significantly increased 1–3 days after infection, and remained around 8.0 log c.f.u. until day 7. At day 14, the bacterial count was significantly decreased compared with day 7. Bars represent the mean bacterial counts. The broken horizontal line represents the detection limit (1.7 log c.f.u. per liver). *P*<0.05; †*P*<0.01.

RESULTS

Liver abscess formation induced by *F. necrophorum* injected into the caudal vein

In preliminary experiments, we found that the liver abscess formation induced by *F. necrophorum* intravenous injection in mice was always accompanied by a persistent decrease in body weight. Two days after infection, the body weight of the 1.6 × 10⁸ c.f.u. group remained significantly lower than that of control mice (23.1 ± 1.1 vs. 25.7 ± 1.4 g per mouse; *P*<0.05 compared with control groups). The 1.6 × 10⁷ c.f.u. group did not exhibit any continuous body weight decrease. Thus, an inoculum containing 1.6 × 10⁸ c.f.u. per mouse was considered sufficient to cause liver abscess formation, and we conducted the remaining experiments using inoculum at 1.6 × 10⁸ c.f.u. per mouse. The change in the number of viable *F. necrophorum* in the liver in the days following infection is shown in Fig. 1. The mean bacterial count in the liver of infected mice significantly increased from day 1 to 3 after infection. The bacterial count remained around 10⁷–10⁸ c.f.u. per liver for 7 days and decreased after 14 days. In order to distinguish co-culture or contamination by other bacteria, we concurrently cultured *F. necrophorum* obtained from the liver on PV Brucella HK agar and blood agar plates. The blood agar culture was conducted under aerobic conditions with a CO₂ incubator. No co-culture or contamination was found on the culture plates, and we determined that all bacterial counts obtained in the experiments were attributable to *F. necrophorum*. The bacterial number and gross abscess formation on day 4 after infection in the liver, blood, lungs, spleen and kidneys are described in Table 1. Gross abscess lesions could be detected only in the liver, and the mean bacterial load was significantly higher in the liver than in the other organs (*P*<0.01 for all). There was preferential abscess formation in the liver throughout the observation period.

The histopathological features of *F. necrophorum* liver abscesses were established by serial macroscopic and microscopic examinations (Fig. 2). On macroscopic
examination, well-demarcated, 2–10 mm diameter yellowish foci with a caseous consistency were observed on the surface of the liver. Adhesion to the surrounding peritoneum was also detected at 14 days after infection. Histopathological examination of livers from infected mice revealed the existence of liver abscesses with necrotic centres and amorphous material admixed with cellular debris (Fig. 2f–j). The size of the abscesses increased from day 1 to day 5 after infection. Neutrophil aggregates were present in the periphery of the necrotic area from day 3 after infection (Fig. 2l, m) and fibrosis accompanied by fibroblast proliferation and infiltration of foam cells was observed from day 7 (Fig. 2n). The neutrophils and macrophages were separated from the necrotic centre by the surrounding layer. In the outermost layer, a granulomatous layer was observed from day 7 (Fig. 2n) and enlarged by day 14 (Fig. 2o), which was considered to correspond to the demarcation against necrotic tissue.

Treatment study

In the 5 mg MTZ kg⁻¹ group, body weight increased during the treatment period and remained significantly higher compared with the untreated group until day 14 (data not shown). At 4 days after inoculation, gross liver abscess formation was not observed for the 5 mg MTZ kg⁻¹ group, although liver abscesses were a feature of infection in all other mice. The change in the number of viable F. necrophorum in the liver of infected mice with MTZ treatment is shown in Fig. 3. Liver bacterial count on day 4 was significantly lower in the 5 mg MTZ kg⁻¹ group compared with the other groups. In the 0.05 mg MTZ kg⁻¹ group, although no reduction in bacterial count or liver abscess eradication was found after MTZ injection, body weight increased 10 days after infection and remained significantly higher than in the untreated group (data not shown).

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**Table 1.** Bacterial load in the liver, blood, lungs, spleen and kidneys of F. necrophorum-infected mice 4 days after inoculation

<table>
<thead>
<tr>
<th>Organ</th>
<th>Gross identification of abscesses (positive/total)</th>
<th>Bacterial recovery from organ (positive/total)</th>
<th>Bacterial load (log₁₀ c.f.u. ml⁻¹ or per organ)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>13/13</td>
<td>13/13</td>
<td>8.16 ± 0.56</td>
</tr>
<tr>
<td>Blood</td>
<td>–</td>
<td>2/13†</td>
<td>0.75 ± 0.84†</td>
</tr>
<tr>
<td>Lungs</td>
<td>0/13†</td>
<td>10/13</td>
<td>3.13 ± 1.76†</td>
</tr>
<tr>
<td>Spleen</td>
<td>0/13†</td>
<td>11/13</td>
<td>3.68 ± 1.91†</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0/13†</td>
<td>11/13</td>
<td>2.68 ± 1.63†</td>
</tr>
</tbody>
</table>

*Results are shown as mean ± SD (n=3).  †P<0.01 compared with the liver.

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**Fig. 2.** Pathological analysis of livers from mice infected with F. necrophorum. Livers were collected 1–14 days after inoculation. (a–e) Photographs of whole liver. Haematoxylin and eosin-stained tissue sections at ×40 (f–j) and ×100 (k–l) magnification. Gross yellowish foci with a caseous consistency were evident from 1 day after infection. The lesion enlarged from approximately 1 to 5–7 mm in diameter.
**DISCUSSION**

*F. necrophorum* is a Gram-negative, rod-shaped anaerobic organism that causes abdominal abscesses and respiratory tract infections in humans and animals (Tadepalli et al., 2009). The organism is classified into four biotypes, two of which are considered pathogenic. Biotype A is called subspecies *necrophorum*, which is frequently encountered in animal infections; biotype B is called subspecies *funduliforme*, which is more likely to be the causative agent in human infections (Shinjo et al., 1991; Hagelskjær Kristensen & Prag, 2000). In cattle, *F. necrophorum* is known to be a common cause of liver abscesses (Tadepalli et al., 2009). In humans, it is a causative organism of Lemierre’s syndrome, a septic thrombophlebitis of the internal jugular vein that typically begins with an oropharyngeal infection (Riordan, 2007; Kuppalli et al., 2012). Although liver abscesses are rare in humans and have been identified in only 2–4% of patients with Lemierre’s syndrome (Riordan, 2007), most of the complications (including liver abscesses) of Lemierre’s syndrome occur via haematogenous infection with multiple abscess lesions. Therapeutic intervention in such cases is still controversial.

Abe et al. (1976) described a murine model of *F. necrophorum* infection by intraperitoneal injection. Their model represented acute abdominal infection by *F. necrophorum*, with a high incidence of liver abscess formation and general lack of bacteraemia. In their model, liver, spleen and lung bacterial counts were approximately equal by day 13 after infection, although abscesses were only observed in the liver. Hill (1977) examined the effects of minocycline and tetracycline using an abdominal infection model of *F. necrophorum* and *Fusobacterium nucleatum* in mice. Antibiotic efficacy was evaluated by the rate of eradication of abdominal abscess lesions, and not by bacterial counts in organs. Because the lesions in this model distribute to multiple organs (including the pleura and peritoneal cavity), it seems difficult to evaluate the effect of antibiotics by bacterial count from each organ.

To our knowledge, we have developed the first animal model of liver abscess caused by *F. necrophorum* using haematogenous infection. Following injection of the bacteria into the blood, the pathogen localized to the liver without forming abscesses on other organs, and sequential bacterial proliferation was only observed in the liver. Bacterial counts were undetectable in other organs 14 days after inoculum (data not shown), and were lower even in liver which gross abscess formations were diminished.

We propose two reasons as to why injection of *F. necrophorum* into the caudal vein resulted in abscess formation only in the liver and not in other organs. First, virulence factors of *F. necrophorum* might be responsible for the preferential liver abscess formation of this bacterium. Among the virulence factors of *F. necrophorum* subsp. *funduliforme*, leukotoxin is a secreted protein that is cytotoxic to hepatocytes, neutrophils and macrophages (Narayanan et al., 2002). Various factors affect leukotoxin production, including subspecies, growth phase and composition of medium, and the inoculation conditions of *F. necrophorum* in our study might have been suitable for the production of high amounts of leukotoxin. Secondly, the caudal vein injection might achieve high accumulation of *F. necrophorum* in the liver for anatomical reasons. We found that a certain high bacterial load is indispensable for producing liver abscess by caudal vein injection. Because of the anatomical priority, caudal vein injection might achieve an initially high bacterial load in the liver that enables *F. necrophorum* to overcome aerobic circumstances.

From these results, we consider that liver abscess formation preferably results from *F. necrophorum* infection via caudal vein injection. Because the haematogenous liver abscess formation quite uniformly progressed with reproducible timing, this model may be suitable for examining the effects of antibiotics at various stages of liver abscess formation.

Furthermore, as most complications in human *F. necrophorum* infection occur via haematogenous infection, our haematogenous liver abscess model might provide certain clues for understanding the partial pathogenesis of the human disease.

In conclusion, we have established a novel murine model of *F. necrophorum* liver abscess via haematogenous infection. This may be useful not only for investigating *in vivo*
antimicrobial activity on anaerobic abscess lesions, but also for investigating *F. necrophorum* pathogenicity in abscess formation.

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**REFERENCES**


