Characteristic features of intracellular pathogenic 
*Leptospira* in infected murine macrophages

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Introduction

Phagocytosis plays a key role in our innate ability to restrict the spread of infectious diseases. Microorganisms internalized by phagocytosis are sequestered in phagosomes, which are initially inapt at killing and degrading pathogens. These phagosomes acquire their microbicidal properties through a complex maturation process involving sequential fusion with endocytic organelles, leading to the formation of phagolysosomes (Stuart and Ezekowitz, 2005). Through evolution, some pathogens such as *Mycobacterium tuberculosis*, *Salmonella* and *Legionella pneumophila* among others, have developed strategies to adapt and survive in their hosts by avoiding the harsh environment of phagolysosomes (Fratti et al., 2003; Kagan et al., 2004; Meena and Rajni, 2010; Jantsch et al., 2011).

*Leptospira* are divided into two major groups: *L. interrogans* sensu lato and *L. biflexa* sensu lato. The former is pathogenic for humans and animals, while the latter is free-living (Picardeau et al., 2008). More than 230 different serovars belonging to 28 serogroups of *L. interrogans* have so far been identified, based on serological examinations. Rodents constitute the main reservoir of *L. interrogans* and asymptptomatically excrete the bacteria in their urine (Bharti et al., 2003). Humans may become infected via contaminated water. The course of human leptospirosis ranges from mild to severe; some forms can be fatal, depending on the serovar of *Leptospira* involved, its virulence and the inoculum size (Levett, 2001; Adler and de la Peña Moctezuma, 2010). Leptospires are highly motile bacteria that are able to penetrate the abraded skin and mucous membranes and rapidly disseminate to other tissues shortly after infection (Ko et al., 2009; Werts, 2010). Although the virulence mechanisms remain largely unknown, several leptospiral outer membrane proteins that interact with mammalian extracellular matrix have been characterized (Ristow et al., 2007; Hoke et al., 2008; Atzingen et al., 2009; Murray et al., 2009; Haake and Matsunaga, 2010). These organisms are clearly invasive (Merien et al., 1997; Barocchi et al., 2002; Li et al., 2007) and the success of their invasion is mainly due to their ability to survive and grow in tissues by escaping innate defence mechanisms. Some studies have suggested that pathogenic leptospires are able to survive in...
host cells such as peritoneal macrophages, Kupffer cells, microglial cells and human peripheral blood monocytes (Cinco et al., 1981; 2006; Marangoni et al., 2000; Li et al., 2010). However, the long-term survival of L. interrogans in murine macrophages has never been investigated.

In this paper, the intracellular trafficking undertaken by non-pathogenic L. biflexa and pathogenic L. interrogans after they have become engulfed through nonopsonic phagocytosis was compared in an attempt to elucidate whether L. interrogans can survive and replicate within murine macrophages. L. interrogans clearance in murine macrophages was delayed and was able to survive. Furthermore, the surviving leptospires replicated and were released to the extracellular milieu.

Results

Delayed clearance of L. interrogans in infected bone marrow-derived macrophages

A previous study showed that L. interrogans (serovar Lai, strain Lai) ingested by murine macrophages was rapidly killed and that bacterial infection had induced the apoptosis of macrophages at 4 h after infection (Li et al., 2010). To clarify the anti-bacterial functions of macrophages upon Leptospira infection, the fates of non-pathogenic L. biflexa and pathogenic L. interrogans (serovar Manilae) after the infection of murine bone marrow-derived macrophages (BMMs) were compared. The phagocytic uptake, bactericidal activity or cell death of the infected BMMs were analysed using immunofluorescence imaging. An inside/outside staining method to distinguish between internalized and extracellular bacteria revealed that most of the leptospires had not been phagocytosed at 10 min post infection (pi); however, at 1 h pi, a large number of leptospires had been internalized in the macrophages, as observed by the presence of leptospires-containing phagosomes (LCPs). During the course of infection, a rapid decrease in intracellular L. biflexa was also observed, compared with that of L. interrogans (Fig. 1A). To better define this time-course of events, the overall levels of macrophage infection were monitored by scoring the number of LCPs that reacted with anti-L. biflexa or anti-L. interrogans antisera. At 1 h after infection, more than 80% of the macrophages contained LCPs for both strains. However, the percentage of macrophages that harboured more than five LCPs was higher in L. biflexa-infected macrophages (~70%) than in L. biflexa-infected macrophages (~40%). At 4 h pi, the percentage of BMMs without LCPs had increased in the L. biflexa-infected macrophages (~34%), whereas ~90% of the L. interrogans-infected macrophages still contained LCPs, suggesting that by 4 h after infection, L. biflexa but not L. interrogans had been cleared in ~14% of the infected macrophages (Fig. 1B). Because anti-Leptospira antisera can detect both intact and degraded leptospires, the infected macrophages were also examined using transmission electron microscopy. The BMMs contained intact leptospires at 1 h after infection with either strain. In contrast, by 4 h pi in the L. biflexa-infected BMMs, about 90% of the macrophages contained no leptospires or degraded leptospires, whereas intact spirochaetes were still seen within the vacuoles in 60% of the macrophages in L. interrogans-infected BMMs (Fig. 2). These results suggested that the degradation of L. interrogans is delayed or partially inhibited in infected BMMs and that the phagocytic pathways between the pathogenic and non-pathogenic leptospires are somehow different. Although cell death has been reported by others (Merien et al., 1997; Jin et al., 2009; Li et al., 2010), we did not detect cell death as determined by the release of lactate dehydrogenase from infected BMMs or microscopic observation (data not shown).

Different kinetics of lysosomal marker recruitment by L. interrogans and L. biflexa

To better understand the interaction of leptospires with the phagocytic pathway, the trafficking of LCPs of L. biflexa or L. interrogans was examined in macrophages using immunofluorescence staining against several endosomal and lysosomal markers. Thirty minutes after infection, ~50% of the intracellular bacteria were found in compartments positive for the early endosomal antigen EEA1 (Fig. 3A). This interaction had decreased at 1 h after infection. Concurrent with this decrease, colocalization with the late endosomal antigen (LAMP1) was observed at 1 h after infection (Fig. 3A). For both strains, no difference in the recruitment of either EEA1 or LAMP1 was observed. In contrast, the colocalization of the lysosomal protease cathepsin D with the L. interrogans-containing phagosomes was significantly delayed at 2 h and 4 h after infection (Fig. 3A and B). Quantitative analysis showed that the difference was pronounced at 2 h, when 34% of the L. interrogans versus 57% of the L. biflexa was colocalized with cathepsin D (P < 0.05). Colocalization with cathepsin D had increased at 4 h for L. interrogans (53%), but continued to lag behind that of the L. biflexa-containing phagosomes (74%). A lower colocalization of L. interrogans with the acidotropic probe LysoTracker Red was observed at 1 h, 2 h and 4 h after infection, compared with the colocalization of L. biflexa and LysoTracker Red (Fig. 3A and C). These results suggested that although the majority of L. interrogans-containing phagosomes undergo normal phagosome maturation, a certain fraction does not recruit lysosomal proteases, such as cathepsin D. These phagosomes may not be acidified either by the inhibition of fusion with lysosomes or by bacterial factor/s capable of inhibiting phagolysosomes acidification.
L. interrogans survives within murine macrophages

Next, whether L. interrogans can survive within murine macrophages was examined. BMMs were infected with either L. biflexa or L. interrogans for 24 h, fixed, and immunostained with anti-Leptospira antisera. As shown in Fig. 4A, the infected BMMs contained a large number of L. interrogans-containing phagosomes. Quantification of the LCPs per macrophage revealed that about half of the macrophages did not harbour LCPs among the L. biflexa-infected BMMs. In contrast, among the L. interrogans-infected BMMs, half of the BMMs contained more than five LCPs (Fig. 4B). Interestingly, extracellular L. interrogans was also observed around some of the infected macrophages, while no extracellular bacteria were observed around the macrophages infected with L. biflexa (Fig. 4C). Because the extracellular bacteria were extensively washed and killed with gentamicin 1 h after infection, this result suggested that there was bacterial exit from L. interrogans-infected macrophages. This bacterial exit was not accompanied by cytotoxicity (Fig. 4D). To determine the differences in long-term survival between the two strains more accurately, BMMs were infected with either L. biflexa or L. interrogans for 2 h, 24 h or 48 h; at each time point, the cell-associated bacterial DNA or bacterial DNA in the supernatant was quantified by real-time PCR. Less than 10 leptospires per 10^3 macrophages were detected in the L. biflexa-infected BMMs or the supernatants at 24 h pi. In contrast, tens of cell-associated and hundreds of extracellular leptospires per 10^3 macrophages were detected in the L. interrogans-infected BMMs until 48 h pi (Fig. 4D). To examine whether the intracellular leptospires were degraded, the infected BMMs were analysed using transmission electron microscopy. Intact spiro-
chaetes were observed in the L. interrogans-infected BMMs, while all the spirochaetes were degraded in the L. biflexa-infected BMMs (Fig. 5). These results suggested that L. interrogans can survive within macrophages and are released into the extracellular space.

L. interrogans replicates within murine macrophages

Next, we examined whether L. interrogans can replicate within macrophages. To quantify the viable intracellular bacteria, cell-associated bacteria from BMMs infected with L. biflexa or L. interrogans for 2 h, 24 h or 48 h were recovered and the amount of viable bacteria was determined after cultivating in Ellinghausen-McCullough-Johnson-Harris (EMJH) broth. About 10% of the intracellular leptospires were recovered as cell-associated leptospires from the L. interrogans-infected BMMs at 24 h pi, and this population increased at 48 h pi. Furthermore, an extracellular population of L. interrogans, but not of L. biflexa, was recovered at 24 h and 48 h pi (Fig. 6). On the other hand, less than 1% of the intracellular bacteria were recovered from the L. biflexa-infected BMMs. These data suggested that L. interrogans can, at least in part, survive and replicate within murine macrophages and that the surviving bacteria are released into the extracellular space.

Discussion

Phagocytosis and concomitant sensing of invading pathogens are two important events of the innate immune response against bacteria. L. interrogans is motile and migrates via a breach in the skin into the bloodstream, where the bacteria are rapidly disseminated and subsequently colonize the liver, lungs and kidneys, which constitute the main target organs of Leptospira (Viriyakosol et al., 2006; Chassin et al., 2009; Monahan et al., 2009). However, the innate immune responses during the primary stage of infection and the manner in which this first line of defence is evaded by pathogenic Leptospira remain obscure. In the present study, we focused on the interaction of non-pathogenic Leptospira and pathogenic Leptospira with macrophages, which play an important role in the innate immune response.
role in the innate immune responses. The clearance of *L. interrogans* is delayed in murine macrophages, showing that *L. interrogans* has a capability to survive and replicate within these cells. Moreover, the surviving leptospires are released to the extracellular compartment, suggesting that this release from macrophages may be a means of escaping the innate immune system and spreading to the target organs.

Several reports have shown that the mouse and human macrophages undergo cell death by *L. interrogans* infection.
Leptospira interrogans survives within murine macrophages and is released to the extracellular compartment. A. Representative confocal images showing infected BMMs after 24 h of infection. Total bacteria were detected using TRITC-labelled anti-Leptospira antibodies (red). Cells were counterstained with TO-PRO-3 (blue) to label the DNA. The merged image with TRITC bacteria, TO-PRO-3 and DIC are also shown. Scale bar, 10 μm.

B. Quantification of LCPs per macrophage after 24 h of infection. Data are the mean ± SD of three independent experiments. At least 100 macrophages were counted for each experiment.

C. BMMs were infected with either L. biflexa or L. interrogans for 24 h, fixed and processed for immunofluorescence. Extracellular bacteria were stained with FITC-labelled anti-Leptospira antibodies (green), which were added before permeabilization with saponin; the total bacteria were stained with TRITC-labelled anti-Leptospira antibodies (red). Cellular and bacterial DNA were visualized using TO-PRO-3 (blue). The merged image with extracellular and total bacteria, and TO-PRO-3 are also shown. Scale bar, 10 μm.

D. Release of macrophage LDH into culture supernatants from infected BMMs at 24 h pi. The Salmonella enterica serovar Typhimurium SL1344 ΔSPI-1 (Salmonella pathogenicity island-1) mutant strain was used as positive control. The values are the mean ± SD of triplicate samples.

E. BMMs were infected with either L. biflexa or L. interrogans for various times and the bacteria in the supernatant and the cell-associated bacteria were quantified using real-time PCR. Data are the mean ± SD of three independent experiments.
tion when using serovar Lai or serovar icterohaemorrhagiae strains (Merien et al., 1997; Jin et al., 2009; Li et al., 2010). In this study, we found that L. interrogans serovar Manilae did not induce murine macrophage cell death. Induction of apoptosis in macrophages has been reported to be correlated with virulence; however, the strain used in this study was highly virulent and can cause a lethal infection in C3H/HeJ mice (data not shown). Therefore, our data suggested that the absence of cytotoxicity is not correlated with virulence. Although the reasons of the different behaviours of macrophages infected with different strains are unknown, our data highlight strain specific differences. Moreover, the discrepancy with previous studies may be attributed to different experimental designs.

A previous report suggested that LCPs undergo normal phagosome maturation based on their colocalization with LAMP1 (Liu et al., 2007). However, the colocalization of bacteria with LAMP1 is not sufficient to conclude that a phagosome undergoes normal maturation. For example, LAMP1 positive/cathepsin D negative vacuoles have been reported in Listeria monocytogenes-infected macrophages. These LAMP1 positive vacuoles were found to be non-acidic and to act as non-degradative compartment allowing the replication of L. monocytogenes (Birmingham et al., 2008). Consequently, the colocalization of leptospires with additional lysosomal markers, such as cathepsin D and LysoTracker, was also examined in the present study. A decrease in the colocalization of L. interrogans with these markers was observed, compared with the colocalization of L. biflexa and the markers. Furthermore, L. interrogans was capable of surviving and replicating within the macrophages. However, further works are needed to understand the role of LAMP1 positive/cathepsin D negative phagosomes during the infection of macrophages with L. interrogans.

Some studies have suggested that pathogenic leptospires are able to survive in host cells by recovering leptospires after short-term infection (up to 6 h) (Cinco et al., 1981; 2006; Marangoni et al., 2000). The long-term survival and replication of L. interrogans was only demonstrated in human monocytes, where leptospires repli-

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**Fig. 5.** Intact L. interrogans at 24 h pi. Electron micrographs of L. biflexa- or L. interrogans-infected BMMs at 24 h pi. Scale bars, 0.5 μm.

**Fig. 6.** Leptospira interrogans replicates within murine macrophages and is released to the extracellular compartment. BMMs were infected with either L. biflexa or L. interrogans for various times, and the bacteria in the supernatant or the cell-associated bacteria were quantified by culturing in EMJH broth and counting using a Petroff-Hauser chamber under dark-field microscopy. Data are the mean ± SD of three independent experiments.
Bacterial strains and growth conditions

L. interrogans serovar Manilae strain UP-MMC-NIID (Koizumi and Watanabe, 2004) was kindly provided by N. Koizumi (National Institute of Infectious Diseases, Tokyo, Japan). L. biflexa serovar Patoc strain Patoc I was purchased from the Institute Pasteur, Paris, France. Both strains were grown at 30°C in EMJH liquid medium. To maintain virulence, strain UP-MMC-NIID was preserved by deep freezing and propagated in specific-pathogen-free C3H/HeJ mice (3 weeks old). Mice were infected intraperitoneally with low-passage-number leptospires, and at 3 days after inoculation, the leptospires were isolated from the kidneys by culturing in EMJH medium and stored at −80°C. Frozen L. interrogans was thawed and passaged less than three times in liquid medium for all the experiments.

The Salmonella enterica serovar Typhimurium SL1344 ΔSPI-1 (Salmonella pathogenicity island-1) mutant strain was used as positive control of cell death induction (van der Velden et al., 2000).

Mice and preparation of macrophages

C57BL/6 mice were obtained from CREA Japan (Tokyo, Japan). BMMs were prepared from the femurs and tibias of C57BL/6 mice by culturing for 10 days in 10% FCS-RPMI 1640 supplemented with 30% L-cell supernatant. All the animal studies used protocols approved by the Animal Care and Use Committee of the University of the Ryukyus (Okinawa, Japan).

Bacterial infection

Bone marrow-derived macrophages were seeded at 1 × 10^5 cells in six-well plates containing 10% FCS-RPMI 1640. The cells were infected with leptospires at an moi of 50 per cell, determined by counting the bacteria using a Petroff-Hauser chamber under dark-field microscopy. The plates were centrifuged at 500 g for 10 min to synchronize the stage of infection. BMMs were then incubated at 37°C in 5% CO₂ for 1 h to trigger phagocytosis. BMMs were extensively washed with pre-warmed RPMI to remove extracellular bacteria and then incubated for 1 h in a medium containing gentamicin (25 μg ml⁻¹) to kill the remaining extracellular bacteria. Thereafter, the infected BMMs were incubated in a gentamicin-free medium.

Immunofluorescence microscopy

Infected BMMs were fixed with 2% paraformaldehyde in PBS overnight at 4°C and processed for immunofluorescence staining. The primary antibodies used were rabbit polyclonal anti-L. interrogans (kindly provided by T. Masuzawa, Chiba Institute of Science, Japan) and anti-L. biflexa (Affinity BioReagents), goat polyclonal anti-EEA1 (N-19; Santa Cruz Biotechnologies), rat monoclonal anti-mouse LAMP1 (1D4B; eBioscience) and goat polyclonal anti-cathepsin D (G-19; Santa Cruz Biotechnologies). The secondary antibodies used were FITC-conjugated, Cy5-conjugated or TRITC-conjugated donkey anti-rabbit; FITC-conjugated donkey anti-rat; and TRITC-conjugated or FITC-conjugated donkey anti-goat antibodies. All the secondary antibodies were obtained from Jackson ImmunoResearch. Cells were counterstained with TO-PRO-3 (Invitrogen) to label the DNA. Samples were observed using a confocal laser scanning microscope (Leica TCS-SPE).

Transmission electron microscopy

The infected BMMs on 14 mm plastic cover sheets were fixed for 2 h in 2.5% glutaraldehyde and postfixed in 1% OsO₄. After fixation, the infected cells were dehydrated through a graded series of ethanol and embedded in Epon 812 (TAAB). Following polymerization of the Epon 812 at 60°C for 3 days, ultra-thin sections were cut using an ultratome (MT-2C, RMC) with a diamond knife. The sections were double stained with uranyl acetate and lead citrate, and examined with an electron microscope (H-7500, Hitachi, University of the Ryukyus, Research Laboratory Center).

Live cell imaging

Bone marrow-derived macrophages were infected with FITC-labelled bacteria. Bacteria were labelled with FITC (0.2 mg ml⁻¹) in carbonate buffer (pH 7.2) for 15 min at room temperature, followed by extensive washing. To examine the colocalization of the leptospires with the lysosomes, BMMs were pre-incubated with 75 nM of LysoTracker Red DND-99 (Invitrogen) for 1 h before infection and viewed using a live cell imaging microscope (Leica AF-6500).

Real-time PCR

One hundred microlitres of culture supernatant or 100 μl of lysed-infected BMMs were used for DNA extraction. Two microlitres of
DNA was added to a mixture containing Brilliant II SYBR Green QPCR master mix (Stratagene, Agilent Technologies) and 0.5 μM of both forward and reverse 16S rRNA gene primers. The primers for *L. interrogans* were described previously (Smythe et al., 2002) and the primers for *L. biflexa* designed in this study were 5′-AGTAGCCGGCCTGAGAG-3′ and 5′-CCCATTGAGC AAGATTC-3′. The number of total bacteria was determined using a standard curve generated by the serial dilution (10^4–10^9) of genomic DNA extracted from *in vitro*-cultivated bacteria. Infected BMMs were processed for DNA extraction before the plate centrifugation to use as a template in the control for DNA extraction and PCR inhibition.

**Lactate dehydrogenase assay**

The lactate dehydrogenase (LDH) activity of the culture supernatants of infected cells was measured by using a CytoTox 96 assay kit (Promega) according to the manufacturer’s protocol.

**Quantitative Leptospira growth assay**

For the quantitative analysis of leptospires survival in the BMMs, BMMs were lysed at the indicated times with 1 ml of distilled water and 100 μl aliquots were used to inoculate 2 ml of EMJH broth. The tubes were incubated at 30°C and after 4–6 days, the number of bacteria was determined by counting using a Petroff-Hauser chamber under dark-field microscopy. To determine the amount of surviving bacteria released into the supernatant, 100 μl of culture supernatant was taken before cell lysis, used to inoculate 2 ml of EMJH broth, and counted as described above. The results are expressed as percentage of recovery bacteria with respect to that of intracellular bacteria at 2 h pi (100%). Serially 10-fold diluted bacteria were inoculated to the EMJH broth to use as control.

**Statistical analyses**

Statistical analyses were performed by the Mann–Whitney *U*-test. Differences were considered significant at *P* < 0.05.

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