Infection of Macrophage-Like Cells by *Legionella* Species That Have Not Been Associated with Disease

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We assessed the ability of *Legionella* species that have not been associated with disease to infect macrophage-like U937 cells. Two of fourteen species tested exhibited a 50% infective dose that was within 1 log unit of that of virulent *L. pneumophila*. Indeed, intracellular CFU of *L. jamestowniensis* and *L. parisiensis* increased 100-fold over a 72-h period. These data indicate that additional legionellae can flourish within phagocytes and therefore, can, if given the opportunity, cause disease.

Since the recognition of *Legionella pneumophila* as the etiologic agent of Legionnaires' disease (11), the genus *Legionella* has grown to include 39 species (16, 31). The legionellae normally inhabit aquatic environments or wet soil, surviving much of the time as intracellular parasites of amoebae and ciliates (25–27, 38, 42, 43, 51, 53, 54, 60). Importantly, however, 19 *Legionella* species have been implicated in human disease (2, 23, 57, 59). Indeed, these bacteria are responsible for a significant percentage of both community-acquired and nosocomial pneumonias (23, 40, 63). *Legionella* infections are also manifest in other ways, including cases of pleurisy, endocarditis, pancreatitis, abscess, wound infection, colitis, peritonitis, pyelonephritis, and cellulitis (8, 20, 23, 29, 36, 47). Finally, the flu-like illness known as Pontiac fever has been associated with increases in antibody titer to several *Legionella* species (22–24). It is well known that Legionnaires' disease is a direct result of the ability of *L. pneumophila* to infect and grow within alveolar macrophages (14, 17, 34). The macrophage infectivity of the other "pathogenic" *Legionella* species is less certain. However, on the basis of studies of *L. dumoffii*, *L. feeleeii*, and *L. micdadei* it is generally assumed that replication within phagocytes is a hallmark of virulent legionellae (21, 39, 42, 44). In contrast, the capacity of the remaining 20 species of *Legionella* to parasitize human cells is completely unknown. Since the pathogenicity of these microbes continues to be the subject of speculation and concern (4, 7, 16, 17, 38, 61, 63), we explored their infectivity for macrophages.

To determine whether legionellae are infective for macrophages, we examined their ability to grow within U937 cells, a human cell line that differentiates into macrophage-like cells when treated with phorbol esters (30). A number of investigators have used these cells for the examination of *L. dumoffii*, *L. feeleeii*, *L. micdadei*, and *L. pneumophila* (1, 6, 15, 26, 35, 37, 42, 44, 45, 48–50, 55). To assess the relative infectivities of strains, 50% infective doses (ID_{50} s) were determined at 72 h postinoculation as previously described (15). For this study, we utilized strains representing 14 species that have not yet been implicated in human disease (Table 1). For comparison, the infectivities of 15 pathogenic species were also determined. With the exception of our *L. pneumophila* 130b and *L. micdadei* Rivera strains, all strains originated from the Centers for Disease Control and Prevention. Prior to inoculation into the

U937 cell monolayers, the bacteria were grown for 48 h on buffered charcoal yeast extract (BCYE) agar at 37°C (18).

In keeping with earlier observations (42, 44, 48), strains of *L. dumoffii*, *L. feeleeii*, *L. micdadei*, and *L. pneumophila* were infective for U937 cells, exhibiting ID_{50} s between 30 and 1,000 bacteria (Table 1). The pathogenic isolates of *L. birminghamensis*, *L. bozemanii*, *L. hackeliae*, *L. longbeachae*, *L. maceachernii*, *L. oakridgensis*, and *L. sainthelensi* also had ID_{50} s of less than 1,000 bacteria. In contrast, strains of *L. cherrii*, *L. cincinnatiensis*, *L. gormanii*, and *L. wadsworthii*, representing the four other species associated with disease, exhibited ID_{50} s that were greater than 10^4 bacteria, suggesting that they are defective for intracellular survival or replication. However, since prolonged plate passage results in the attenuation of *L. pneumophila* (13, 14, 17, 48) and since the passage history of these strains is unknown (1a), they could have originally been capable of parasitizing phagocytic cells. In an attempt to resolve this issue, the various isolates were tested for their ability to grow on BCYE agar supplemented with 100 mM NaCl. Among *L. pneumophila* strains, salt sensitivity is associated with the virulent or infective phenotype whereas salt tolerance is a marker for laboratory attenuation (13, 52). With the exception of the *L. dumoffii* strain, all of the infective legionellae tested were, as expected, salt sensitive (Table 1). Interestingly, the noninfective strains of *L. cherrii*, *L. cincinnatiensis*, *L. gormanii*, and *L. wadsworthii* were not salt resistant; i.e., their efficiencies of plating on the NaCl-containing medium were reduced at least 10,000-fold from their efficiencies of plating on standard BCYE agar. Therefore, if one accepts salt resistance as a general indicator of laboratory attenuation, then the inability of these four strains to infect U937 cells cannot simply be ascribed to prolonged or inappropriate plate passage. Alternatively, as the *L. dumoffii* strain suggests, the correlation between the salt sensitivity and infectivity phenotypes might not be complete when all legionellae are considered. In any case, our data clearly indicate that most representatives of the pathogenic species of *Legionella* do replicate within macrophage-like cells.

Among the legionellae that have not been associated with disease, three levels of macrophage infectivity were observed. First, strains of *L. jamestowniensis* and *L. parisiensis* displayed ID_{50} s that were within 1 log unit of that of *L. pneumophila* 130b, indicating that they are quite capable of growing within U937 cells (Table 1 shows the results for one of two representative experiments). Second, the *L. quinlivanii* and *L. santarosensis* isolates had ID_{50} s of approximately 3,000 bacteria, a characteristic that could also be associated with full infectivity.

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TABLE 1. Infectivities of *Legionella* species for U937 cells

Species	Strain	Implication in disease (reference[s])	Log ₁₀ ID ₅₀ ^a	Plating efficiency on BCYE-NaCl ^b
<i>L. adelaidensis</i>	1762-AUS-E	No (3)	4.27 ± 0.15	S
<i>L. birminghamensis</i>	1407-AL-H	Yes (62)	2.76 ± 0.28	S
<i>L. bozemanii</i>	WIGA	Yes (9)	2.75 ± 0.34	S
<i>L. brunensis</i>	441-1	No (61)	4.04 ± 0.48	S
<i>L. cherrii</i>	O RW	Yes (10, 23)	4.70 ± 0.28	S
<i>L. cincinnatiensis</i>	72-OH-H	Yes (58)	5.11	S
<i>L. dumoffii</i>	NY 23	Yes (9)	2.76 ± 0.28	R
<i>L. erythra</i>	SE-32A-C8	No (10)	4.43 ± 0.38	S
<i>L. feeleei</i>	WO-44C	Yes (33)	<3.0	S
<i>L. gormanii</i>	LS-13	Yes (10, 28)	4.22 ± 0.34	S
<i>L. gratiana</i>	1242	No (7)	5.01	ND
<i>L. hackeliae</i>	Lansing 2	Yes (10)	2.22 ± 0.21	S
<i>L. israelensis</i>	Bercovier 4	No (5)	4.90 ± 0.38	R
<i>L. jamestowniensis</i>	JA-26-G1-E2	No (10)	2.01 ± 0.11	S
<i>L. londiniensis</i>	1477	No (16)	5.27 ± 0.34	R
<i>L. longbeachae</i>	Long Beach 4	Yes (41)	1.93 ± 0.13	S
<i>L. maceachernii</i>	PX-1-G2-E2	Yes (10)	<2.9	ND
<i>L. micdadei</i>	Rivera	Yes (32, 44)	1.57 ± 0.36 ^c	S
<i>L. moravica</i>	316-36	No (61)	4.51 ± 0.34	S
<i>L. oakridgensis</i>	OR-10	Yes (46, 56)	<2.8	S
<i>L. parisiensis</i>	PF-209C-C2	No (10)	2.30 ± 0.36	S
<i>L. pneumophila</i>	130b	Yes (11, 15)	1.64 ± 0.28	S
<i>L. quinlivanii</i>	1442-AUS-E	No (4)	3.51 ± 0.35	S
<i>L. rubrilucens</i>	WA-270A-C2	No (10)	5.49 ± 0.15	S
<i>L. sainthelensi</i>	Mount St. Helens 4	Yes (2, 12)	<2.4	ND
<i>L. santarosensis</i>	SC-63-C7	No (10)	3.47 ± 0.34	ND
<i>L. spiritensis</i>	Mount St. Helens 9	No (10)	4.20 ± 0.28	ND
<i>L. steigerwaltii</i>	SC-18-C9	No (10)	>6.2	S
<i>L. wadsworthii</i>	81-716	Yes (19)	4.70 ± 0.28	S

^a The ID₅₀ is expressed as a log value ± the standard deviation (expressed as a log value). The standard deviations for *L. cincinnatiensis* and *L. gratiana* are not presented because no ID₇₅s were obtained.

^b R, equal plating efficiency on BCYE and BCYE containing 100 mM NaCl. S, significantly reduced plating efficiency on BCYE containing 100 mM NaCl. ND, not determined.

^c Data from reference 44.

Finally, representatives of *L. adelaidensis*, *L. brunensis*, *L. erythra*, *L. gratiana*, *L. israelensis*, *L. londiniensis*, *L. moravica*, *L. rubrilucens*, *L. spiritensis*, and *L. steigerwaltii* had ID₅₀s that were greater than 10⁴ bacteria, suggesting that they are impaired for replication within U937 cells. With the exception of the *L. israelensis* and *L. londiniensis* strains, all of these legionellae were sensitive to NaCl (Table 1).

To further define the macrophage infectivities of species not associated with disease, four representative strains were compared with *L. pneumophila* 130b for growth kinetics within U937 cells (Fig. 1). As in previous studies (45, 49), the virulent *L. pneumophila* strain underwent 3 to 4 log units of growth within a 72-h time period. *L. parisiensis* PF-209C-C2 and *L. jamestowniensis* JA-26-G1-E2, the two strains which had ID₅₀s comparable to that of the *L. pneumophila* control, also grew well (Fig. 1A and B). Despite replication rates that appeared, at times, to be lower than that of strain 130b, their numbers of bacteria increased 100-fold. Whereas the *L. parisiensis* and *L. pneumophila* isolates initiated multiplication without any significant delay, the *L. jamestowniensis* organism displayed a 24-h lag. Like strains of *L. pneumophila*, these *L. parisiensis* and *L. jamestowniensis* bacteria did not replicate in the serum-supplemented RPMI medium in the absence of U937 cells (data not shown). Taken together, these observations confirm that strains of *L. parisiensis* and *L. jamestowniensis* productively infect human phagocytes. In contrast, the number of CFU of *L. quinlivanii* 1442-AUS-E within infected monolayers did not increase with time, indicating that this organism, though capable of intracellular survival, is unable to replicate within mononuclear cells (Fig. 1C). Finally, *L. rubrilucens* WA-270A-C2, a

strain that had a very high ID₅₀, also survived poorly within the U937 cell monolayers (Fig. 1D).

Our observations with strains of *L. adelaidensis*, *L. brunensis*, *L. erythra*, *L. gratiana*, *L. israelensis*, *L. londiniensis*, *L. moravica*, *L. quinlivanii*, *L. rubrilucens*, *L. spiritensis*, and *L. steigerwaltii* suggest that a number of legionellae are unable to survive within human phagocytes. Although an inability to infect macrophages diminishes the likelihood that these strains could cause disease, it does not completely eliminate it. Indeed, a strain of *L. anisa* which is unable to flourish within U937 cells has been associated with Pontiac fever (26). Similarly, the salt-sensitive strains of *L. cherrii*, *L. cincinnatiensis*, *L. gormanii*, and *L. wadsworthii* employed in this study appeared unable to grow within the macrophage cell line. Since intracellular infection plays a central role in serious forms of legionellosis, the ability of *L. parisiensis* and *L. jamestowniensis* bacteria to infect macrophages suggests that they could, if given the opportunity, cause disease. This conclusion was tempered by the fact that virulence is multifactorial and involves more than a capacity to productively infect macrophage hosts. Clearly, a demonstration of the ability of these strains to cause disease in experimental animals would reinforce our prediction. Since the *L. parisiensis* strain was isolated from a cooling tower and the *L. jamestowniensis* microbe originated from wet soil (10), human exposure to these types of potential pathogens is possible.

In summary, strains from 11 of 15 pathogenic *Legionella* species were able to replicate within U937 cells, indicating that intracellular infectivity is common among the disease-causing legionellae. In contrast, representatives of only 2 of the 14

FIG. 1. Replication of *Legionella* species within U937 cells. Monolayers ($n = 4$) were inoculated with 10^5 to 10^6 CFU of either *L. pneumophila* 130b (●) or one of the "nonpathogenic" *Legionella* species (○), and after various incubation periods, the numbers of viable intracellular bacteria were determined by plating aliquots on BCYE agar. The species tested included *L. parisiensis* (A), *L. jamestowniensis* (B), *L. quinlivanii* (C), and *L. rubrilucens* (D). Since inoculation of the U937 cell monolayers includes a 2-h entry phase and several washes, the $t = 0$ samples were actually obtained about 3 h after the bacterium-host cell interactions began. In panels B and C, the differences in CFU between strains at $t = 0$ are a reflection of the differences in the inoculum sizes. Most points generally represent the mean CFU recovered, and the vertical bars indicate the standard deviations. The 48- and 72-h datum points for *L. rubrilucens* (D) are estimates, because the numbers of CFU obtained on the agar plates were outside the easily countable range.

species that have not been implicated in disease were infective for a macrophage cell line. To support or modify our conclusions, which were necessarily based upon an examination of one strain per species, it will be important to (quickly) test each new isolate for its infectivity. For example, the fact that one isolate of a species is noninfective does not mean that all strains within that species are noninfective. Nevertheless, our observation that strains of *L. parisiensis* and *L. jamestowniensis* can infect human cells indicates that they, and perhaps other environmental isolates, should be viewed as potential pathogens.

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