INFECTION AND IMMUNITY, May 1994, p. 2111–2114
0019-9567/94/$04.00+0

Vol. 62, No. 5

Cytotoxicity of Extracellular *Legionella pneumophila*

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Received 4 October 1993/Returned for modification 1 December 1993/Accepted 9 February 1994

***Legionella pneumophila*, the causative agent of Legionnaires' disease and Pontiac fever, is known to produce a cytopathic effect on macrophages. The capacity of extracellular *L. pneumophila* to mediate toxicity for guinea pig peritoneal macrophages and J774 mouse macrophages was assessed. Extracellular organisms were found to be capable of mediating toxicity; however, toxic activity appeared to require close proximity with the mononuclear cell surface. Serogroup 1 strains grown on supplemented Mueller-Hinton agar exhibited variable expression of toxic activity. One strain positive on supplemented Mueller-Hinton agar was cytotoxic and unable to replicate in J774 macrophages but remained virulent for guinea pigs at high doses.**

Legionella pneumophila is a facultative intracellular parasite associated with both community-acquired and nosocomial pneumonia. Early studies by Winn et al. (18) on the pathology of the lung suggested that the lysis of the inflammatory exudate and the infarct-like necrosis of the alveolar wall may be due to the production of a toxin. While several toxins have been identified as potential virulence factors (1, 6, 8, 11, 15), the role that these toxins play in the virulence of *L. pneumophila* remains undefined. More recently, interest in the pathogenesis of infections caused by *L. pneumophila* has centered on the ability of *L. pneumophila* to survive and multiply in phagocytic cells. It has been generally assumed that the histopathologic lesions in the lungs during *L. pneumophila* infections are due to the intracellular multiplication of the organism (17). However, we have observed cytotoxic activity by virulent organisms in the absence of demonstrable phagocytosis of the organisms by guinea pig peritoneal macrophages. Thus, experiments were conducted to (i) determine whether *L. pneumophila* is able to exert its cytopathic effect on mononuclear phagocytes in an extracellular environment and (ii) examine the ability of serogroup 1 *L. pneumophila* isolates to produce toxicity following culture on supplemented Mueller-Hinton (SMH) agar. The characteristics of SMH agar-grown organisms are of particular interest since previous studies have shown that SMH agar acts as a selective medium for growth of organisms that are avirulent for guinea pigs (2, 3).

L. pneumophila serogroup 1 strain Philadelphia 2 was obtained from the Centers for Disease Control, Atlanta, Ga. Virulent and avirulent cultures of the Philadelphia 2 strain were derived as previously described (2, 3) and maintained on charcoal-yeast extract (CYE) agar. Two other serogroup 1 isolates, designated 474-39 and 766-1, were obtained from the University of Iowa Hospitals and Clinics Special Microbiology Laboratory, Iowa City, Iowa. These strains were originally isolated from clinical specimens and maintained on CYE agar. In addition, avirulent SMH agar-positive cultures of these strains were derived by selecting isolated colonies on SMH agar and then subjecting them to two successive batch passages on SMH medium. After this selection process, these strains were grown on CYE agar for use in the experiments described here. Unstimulated guinea pig peritoneal macrophages and J774 mouse macrophages were maintained as described previously (9). Briefly, resident peritoneal macrophages were harvested from outbred guinea pigs by peritoneal lavage and cultured in monolayers. The J774 cell line was a gift from Mary Wilson, Department of Internal Medicine, University of Iowa, and was maintained in monolayer culture. The origin of the J774 cell line has been described previously (14).

During previous (unpublished) experiments in our laboratory, virulent Philadelphia 2 strain organisms were opsonized with specific antibody and coincubated with guinea pig peritoneal macrophages for up to 6 h at a relatively high multiplicity of infection (bacterium-to-macrophage ratio, 1,000:1). Under these conditions, very few, if any, organisms were detected intracellularly by transmission electron microscopy; however, the majority of the macrophage monolayer was destroyed. This initial observation led to the hypothesis that an intracellular environment is not required for induction of cytotoxic activity by *L. pneumophila*. In the present study, to test this hypothesis, guinea pig peritoneal macrophages and J774 mouse macrophages were treated with the microfilament inhibitor cytochalasin D to block phagocytosis, and then the ability of extracellular *L. pneumophila* to induce cytotoxicity was assessed. Mononuclear cells were washed two times with antibiotic-free tissue culture medium (RPMI 1640) containing 20% heat-inactivated fetal calf serum and cultured in antibiotic-free medium for a minimum of 1 h before use. One set of mononuclear cells was treated with cytochalasin D (1 μg/ml; Sigma Chemical Co., St. Louis, Mo.) for a minimum of 1 h before addition of the bacteria. Confocal microscopy (not shown) was carried out with the J774 cell line to ensure that treatment with cytochalasin D effectively inhibited phagocytosis. In addition, a previous study has shown that cytochalasin D at 1 μg/ml effectively inhibits phagocytosis of *L. pneumophila* by guinea pig and rat alveolar macrophages (4). Control experiments were also carried out to confirm that cytochalasin D at this concentration is not itself toxic for the mononuclear cells (data not shown). Virulent and avirulent Philadelphia 2 organisms were suspended in antibiotic-free tissue culture medium containing, when appropriate, cytochalasin D (1 μg/ml) and heat-inactivated rabbit antiserum to serogroup 1 (Philadelphia 2) organisms (10). This antiserum has a microagglutination titer of 1/256 and was used for opsonization at a 1/450 dilution. Organisms were added to the mononuclear cells at a bacterium-to-cell ratio of 300:1. (Typically, 2.5×10^6 to 1×10^7 CFU were added to wells containing 8×10^3 to 3×10^4 macrophages.) The macrophages were coincubated with

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TABLE 1. Effect of cytochalasin D on cytotoxic activity of *L. pneumophila* for mononuclear cells[a]

Organism	% Viable[b]			
	Cytochalasin D treated		Untreated	
	GP cells	J774 cells	GP cells	J774 cells
Virulent Philadelphia 2	100 ± 0	82 ± 1	100 ± 0	87 ± 2
Virulent Philadelphia 2 + Ab	48 ± 0	51 ± 6	50 ± 4	68 ± 3
Avirulent (SMH+) Philadelphia 2	NT	100 ± 0	NT	99 ± 1
Avirulent (SMH+) Philadelphia 2 + Ab	NT	99 ± 1	NT	98 ± 1
None	99 ± 1	99 ± 1	100 ± 0	100 ± 0

[a] Abbreviations: GP, guinea pig; Ab, antibody; SMH+, SMH agar positive; NT, not tested. Coincubation times were 2 h for guinea pig cells and 3.5 h for J774 cells. The bacterium-to-cell ratio was 300:1.

[b] Data represent the means ± standard errors of two to four determinations.

organisms until signs of cytotoxicity (cell rounding and vacuolization) were readily apparent (at 2 h with guinea pig cells and 3.5 h with J774 cells). The mononuclear cells were then washed once with Hanks' balanced salt solution, and cell viability was determined by vital staining with an Erythrosin B (Fisher Scientific Co., Fair Lawn, N.J.) solution (0.05% [wt/vol] in potassium phosphate-buffered saline [pH 7.6; KPBS]) and then by observation under a model CK2 inverted microscope (Olympus Optical Co., Ltd., Tokyo, Japan). For cell viability determinations, a minimum of 100 consecutive mononuclear cells from one representative field were examined from each monolayer. The results are shown in Table 1. No significant differences were observed in the degree of cytotoxicity obtained with mononuclear cells challenged in the presence and absence of cytochalasin D. In addition, when the organisms were treated with opsonizing antibody, toxicity for both treated and untreated macrophages was enhanced. These results indicate that cytotoxic activity may be carried out when the organisms are present extracellularly; however, toxic activity appears to be enhanced by close contact with the macrophage surface, since cytotoxicity was augmented by opsonizing antibody. Furthermore, in the presence of antibody, the multiplicity of infection required to elicit cytotoxic activity for guinea pig peritoneal macrophages after 24 h of incubation was 0.1 organism per macrophage. In contrast, in the absence of antibody, cytotoxicity was not observed after 24 h of incubation until the multiplicity of infection was increased to 100 organisms per macrophage (data not shown). Toxicity was not observed with avirulent (SMH agar-positive) Philadelphia 2 organisms (Table 1).

Since cytotoxic activity in the presence of cytochalasin D was significantly enhanced by treating the organisms with opsonizing antibody, suggesting a requirement for contact with the macrophage surface, unopsonized organisms and mononuclear cells were coincubated in tissue culture medium on opposite sides of a permeable membrane to confirm that physical separation would inhibit toxicity. This separation was achieved through the addition of Millicell-HA culture plate inserts (Millipore, Inc., Bedford, Mass.) containing 0.45-μm-pore-size mixed cellulose ester membranes to wells of a 24-well tissue culture plate which had been seeded with 3×10^4 J774 macrophages. The level of tissue culture medium in the wells was maintained below the level of the top of the Millicell insert to prevent direct mixing of organisms with macrophages. However, the 0.45-μm-pore-size membrane allowed free movement between the compartments of the tissue culture medium and any secretory products produced by the macrophages or organisms. Organisms (5×10^8 CFU) were suspended in KPBS and added to the Millicell inserts or directly to the macrophage monolayers at a bacterium-to-macrophage ratio of ca. 16,000. This large bacterium-to-macrophage ratio was necessary to achieve detectable toxicity in a reasonably short period of time in the absence of opsonizing antibody. Preliminary experiments demonstrated that unopsonized, virulent organisms are capable of producing the cytotoxic effect on both J774 and guinea pig macrophages if the multiplicity of infection or coincubation time is increased (data not shown). The J774 cells were coincubated with organisms for 3 h, the Millicell chambers were removed, and cell viability was determined by Erythrosin B staining as described previously. Triplicate macrophage monolayers were infected. When the organisms were allowed direct contact with the macrophages, a 61% ± 2% (mean ± standard deviation) cell viability was observed. The cell viability rose to 96% ± 3% (mean ± standard deviation) when the organisms were added to the insert rather than directly to the monolayer, indicating that cytotoxic activity by the organisms is inhibited by physical separation of the organisms from the target cells. These results strongly suggest that cytopathic activity by *L. pneumophila* requires direct contact with the macrophage cell surface. However, from these experiments, the possibility that close proximity to the macrophage surface may be sufficient for toxicity cannot be excluded.

TABLE 2. Cytotoxicity of CYE and SMH agar-grown serogroup 1 isolates for J774 cells[a]

Strain	% Viable[b]	
	CYE agar culture	SMH agar culture
Philadelphia 2	0 ± 0	99 ± 2
474-39	1 ± 1	1 ± 1
766-1	0 ± 0	82 ± 2

[a] Coincubation time was 18 h. J774 cells were cytochalasin D treated; organisms were opsonized with specific antiserum. The bacterium-to-cell ratio was 1,000:1.

[b] Data represent the means ± standard deviations of triplicate macrophage monolayers and are representative of three separate experiments.

The next experiments were performed to determine (i) if cytotoxic activity by serogroup 1 isolates other than the Philadelphia 2 strain could also be carried out in an extracellular environment and (ii) if other SMH agar-positive, serogroup 1 isolates also do not produce this effect. These experiments were carried out with cytochalasin D-treated J774 cells and opsonized organisms essentially as described above for the test of the effect of cytochalasin D on the cytotoxic activity of *L. pneumophila* for mononuclear cells, with two modifications. The bacterium-to-macrophage ratio was increased to 1,000:1 (2×10^7 organisms per 2×10^4 J774 cells), and the coincubation time was increased to 18 h to detect low-level toxicity. The results of these experiments are given in Table 2. As was demonstrated with the Philadelphia 2 strain, isolates 474-39 and 766-1, when grown on CYE agar, both possess cytotoxic activity which results in destruction of cytochalasin D-treated J774 monolayers. In contrast to the Philadelphia 2 strain, however, strains 474-39 and 766-1 maintained a level of cytotoxicity after passage on SMH agar. Cytotoxic activity by strain 474-39 grown on SMH agar was equivalent to that observed with corresponding organisms grown on CYE agar (both strains produced a 99% loss of macrophage viability over 18 h of coincubation). Cytotoxic activity by strain 766-1 grown on SMH agar was reduced compared with that of the corre-

TABLE 3. Virulence of strain 474-39 organisms for guinea pigs[a]

Growth medium	Dose (no. of organisms)	Lethality (no. of animals dead/ total no.)
SMH agar	10^9	4/4[b]
	10^7	0/4
CYE agar	10^9	3/3[c]
	10^7	3/3[c]

[a] Organisms were administered intraperitoneally.
[b] Mean time to death, approximately 6 h.
[c] Death was observed at 2 to 3 days.

sponding CYE agar-grown organisms (18% loss of macrophage viability compared with a 100% loss of viability, respectively). Therefore, serogroup 1 isolates grown on SMH agar appear to exhibit variable expression of cytotoxic activity. The factors responsible for the observed differences among these strains have not been determined. However, strains 474-39 and 766-1 are recently acquired patient isolates and have been minimally passaged on laboratory medium, while the avirulent strain Philadelphia 2 has been cultivated on artificial medium for a considerable length of time. It may be possible that loss of cytotoxic activity occurs gradually over successive passages on laboratory medium.

Since strain 474-39 fully retained its ability to produce toxicity following passage on SMH agar, these SMH agar-positive organisms were examined for two other important virulence characteristics, lethality for guinea pigs and replication within macrophages. For the guinea pig experiments, male Hartley strain guinea pigs, weighing 400 to 500 g, were obtained from Sasco, Inc., Omaha, Nebr. A dose of 10^9 CFU of the SMH agar-positive strain 474-39 (suspended in KPBS) was injected intraperitoneally into each of four animals. In addition, four guinea pigs were challenged with 10^7 CFU (a dose 1 to 2 logs greater than the usual 50% lethal dose for virulent *L. pneumophila* strains). The results are given in Table 3. Those animals receiving 10^9 organisms died rapidly (mean time to death, approximately 6 h). This rapid onset of death is in contrast to what is typically observed when guinea pigs are challenged with CYE agar-grown Philadelphia 2 organisms (2) as well as other virulent strains. Normally, death is observed in 2 to 3 days after specific signs of illness, including weight loss, watery eyes, and ruffled fur (2), occur. The test animals receiving 10^7 CFU of SMH agar-positive strain 474-39 organisms exhibited no signs of illness and remained healthy. SMH agar-cultured (avirulent) Philadelphia 2 organisms do not induce illness in guinea pigs when given at doses as high as 10^{10} CFU (2). When strain 474-39 was grown on CYE agar to maintain its virulence, guinea pigs injected with either 10^7 or 10^9 organisms died within 2 to 3 days and exhibited symptoms typically associated with legionella infections.

The capacity of SMH agar-positive strain 474-39 organisms to replicate within macrophages was then assessed. The J774 mouse macrophage cell line was utilized for these assays. Macrophages were washed three times with antibiotic-free tissue culture medium and cultured in antibiotic-free medium containing 20% heat-inactivated fetal calf serum for a minimum of 1 h before use. Forty-eight-hour cultures of *L. pneumophila* were suspended in KPBS and opsonized with heat-inactivated polyclonal antiserum for 30 min at 37°C before addition to the monolayers. Organisms were added to the macrophages at a ratio of 10:1 and incubated in a humidified atmosphere of 5% CO_2 without agitation. The monolayers were not washed after infection. The culture supernatants

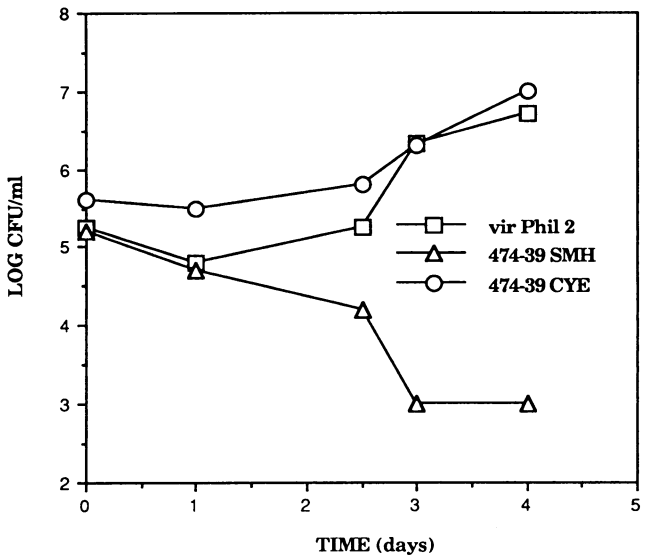

FIG. 1. Growth of SMH agar-passaged strain 474-39 organisms (474-39 SMH) in J774 mouse macrophages. Organisms were opsonized with specific antiserum and added to the monolayers at a ratio of 10:1. CYE agar-grown strain 474-39 (474-39 CYE) and Philadelphia 2 (vir Phil 2) organisms were included as controls to demonstrate that this cell line does support *L. pneumophila* growth. The results shown are representative of two separate experiments. One monolayer was infected per strain per experiment.

were sampled daily by removing a 100-μl portion, and the CFU of *L. pneumophila* per ml of tissue culture medium was determined by serial dilution and plating, in duplicate, on CYE agar. Since *L. pneumophila* is unable to replicate in standard tissue culture medium, any increase in CFU in the medium is the result of intracellular multiplication. As shown in Fig. 1, no increase in CFU was observed for strain 474-39 organisms passaged on SMH agar, indicating that these organisms were unable to replicate within the J774 cells. Virulent Philadelphia 2 and CYE agar-passaged 474-39 cultures were included as controls to demonstrate that J774 cells are indeed able to support the growth of *L. pneumophila*. For these CYE agar-passaged strains, a 1.4-log increase in CFU was observed over 4 days of coincubation.

In the animal experiments described above with strain 474-39, the rapid onset of death and dependence on a relatively large dose of organisms suggest that death was more likely the result of toxicity than of an infectious process. The inability of this strain to replicate within macrophages further supports this possibility. However, from these experiments, we are unable to conclude that this symptomatology and this mortality in guinea pigs are related to the macrophage toxicity we have observed. Fields et al. (5) have described a *Legionella anisa* strain isolated from an indoor fountain implicated as the infectious reservoir in an outbreak of an acute, nonpneumonic, self-limited febrile illness known as Pontiac fever. The characteristics of this *L. anisa* isolate are similar to those of the SMH agar-positive strain 474-39 organisms described above. Both strains are unable to replicate within macrophages and produce an acute illness when given intraperitoneally to guinea pigs at relatively high doses but do not cause illness in guinea pigs when given at moderate doses. Fields et al. (5) have suggested the possibility that the outcome of Pontiac fever and not Legionnaires' pneumonia in individuals following exposure

to this *L. anisa* isolate was due to its inability to replicate within macrophages. Unfortunately, it was not determined whether this *L. anisa* strain is capable of mediating cytotoxicity; however, its effect on guinea pigs is also consistent with toxicity.

The specific toxin responsible for the cytotoxic effects observed in these experiments has not been identified. A metalloprotease that is cytotoxic for Chinese hamster ovary cells has been described previously (11, 13). However, recent studies (16) have shown that the metalloprotease is not required for intracellular survival or cytotoxicity for HL-60 cells. In addition, a metalloprotease-deficient mutant is fully cytotoxic for J774 macrophages (data not shown). Friedman et al. (6) reported the isolation of a low-molecular-weight toxin that was cytotoxic for Chinese hamster ovary cells, and Hedlund (8) reported the isolation of a low-molecular-weight toxin that was active against mouse macrophages. Since both of these toxins are found in culture supernatant fluids, it appears unlikely they are responsible for the cytotoxicity observed in the present studies. The legiolysin reported by Rdest et al. (15) is not cytotoxic for Vero or Chinese hamster ovary cells (7) and also appears to be an extracellular product. A membrane bound ADP-ribosyltransferase has been reported by Belyi et al. (1), but its activity on intact macrophages has not yet been determined. McCusker et al. (12) reported that *L. pneumophila* inhibited protein synthesis in Chinese hamster ovary cells, but the specific toxin responsible for this activity has not been identified. It is unlikely that endotoxin plays a major role in the cytotoxicity observed in these studies because formalin-killed cells were not cytotoxic (data not shown). In addition, Wong et al. (19) have shown that endotoxin isolated from *L. pneumophila* has relatively low toxicity compared with endotoxin isolated from other gram-negative bacteria. Additional studies will be required to determine whether the macrophage toxicity observed in the current studies is associated with any of the previously reported toxins.

To our knowledge, this is the first report demonstrating that cytotoxicity may occur in the absence of intracellular replication. In addition, the ability of an *L. pneumophila* strain to produce illness in guinea pigs in the absence of the ability to replicate within macrophages has not been described previously. Future research on the pathogenesis of this organism must consider the extracellular activities of this organism in addition to its capacity for intracellular survival.

Finally, this study has demonstrated the utility of the J774 mouse macrophage cell line for studying the cytopathic effect of *L. pneumophila* on macrophages. J774 cells are known to exhibit the adherent, cytologic, and phagocytic properties of peritoneal macrophages (14), and since these cells are able to support the growth of *L. pneumophila*, they may also be useful in future studies concerning mechanisms of intracellular survival. Additional experiments are needed to compare the efficiency of growth and the survival in these cells with those characteristics of the established human cell lines. The expense and difficulty of obtaining large numbers of primary macrophages often demand that suitable cell lines be identified and utilized, and it is expected that the J774 cells will continue to be a useful tool in *Legionella* research.

We thank Mary Wilson for providing the J774 cell line.

This work was supported by Public Health Service grant AI 26523 from the National Institutes of Health.

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