Cytolytic and Phospholipase C Activity in *Legionella* Species

By WILLIAM B. BAINE

Departments of Internal Medicine and Microbiology, Southwestern Medical School and Southwestern Graduate School of Biomedical Sciences, The University of Texas Health Science Center at Dallas, 5323 Harry Hines Boulevard, Dallas, Texas 75235, USA

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To examine one possible mechanism of damage to leucocytes and tissue cells in legionellosis, seven species of *Legionella* were examined for cytolytic activity and for elaboration of phospholipase C, an enzyme that can damage mammalian cell membranes. Cytolysis was assessed using erythrocytes in agar. Phospholipase C was assayed by release of *p*-nitrophenol from *p*-nitrophenylphosphorylcholine and of tritiated phosphorylcholine from L- α -dipalmitoyl-[choline-methyl-³H]phosphatidylcholine. *L. pneumophila*, *L. bozemanii*, *L. micdadei*, *L. dumoffii*, *L. gormanii*, *L. longbeachae* and *L. jordanis* all lysed dog red blood cells, which have a high ratio of membrane phosphatidylcholine to sphingomyelin. The same strains hydrolysed varying amounts of *p*-nitrophenylphosphorylcholine; *L. bozemanii* exhibited the greatest activity. *L. pneumophila*, *L. bozemanii*, *L. dumoffii*, *L. longbeachae* and *L. jordanis*, but not *L. micdadei*, released tritiated phosphorylcholine from labelled substrate. These results indicate that several species of *Legionella* possess cytolytic capability; exotoxins with phospholipase C activity may play a role.

INTRODUCTION

Legionella pneumophila and other species of *Legionella* are aetiological agents of pneumonia in man (Meyer, 1983). Despite rapid progress since 1976 in delineating the epidemiology and clinical manifestations of legionellosis, much remains to be learned about the mechanisms of pathogenesis at the tissue and cellular levels in *Legionella* infections.

Many pathogenic bacteria elaborate cytolytins – exotoxins that damage host-cell membranes *in vitro* (Alouf, 1977). In certain bacterial infections some of these cytolytic toxins may also play a role in pathogenesis *in vivo*. Cultures of *L. pneumophila* lyse mammalian erythrocytes in agar medium (Baine *et al.*, 1979a). Guinea-pig erythrocytes, in which the cell membrane has a high ratio of phosphatidylcholine to sphingomyelin, are particularly susceptible (Baine *et al.*, 1979a). Cultures of *L. pneumophila* also produce an opaque zone in agar medium containing egg yolk (Baine *et al.*, 1979a), a reaction resembling that produced by *Clostridium perfringens* on egg-yolk agar and attributed to clostridial α -toxin (Smith *et al.*, 1980). The α -toxin is a phospholipase C (EC 3.1.4.3), which hydrolyses phosphatidylcholine to 1,2-diacylglycerol and phosphorylcholine (Möllby, 1978).

This report extends earlier observations on haemolytic activity and digestion of egg yolk exhibited by *L. pneumophila* to six additional species of *Legionella* and assesses phospholipase C activity in *Legionella* species by means of specific biochemical assays. In the present study haemolysis, digestion of egg yolk and phospholipase C activity are evaluated using *Legionella* cultures or suspensions of bacterial cells as a prelude to future attempts to purify the putative toxins and enzymes from culture supernates or lysates.

Abbreviations: BYE, buffered yeast extract medium; ABYE, augmented BYE; DBYE, dialysed BYE.

METHODS

Bacterial strains. Representative strains of *L. pneumophila* serogroups 1 (Philadelphia 1, Pontiac 1), 4 (Rockport 1), 5 (Dallas 1E) and 6 (Chicago 2), *L. bozemanii* serogroup 1 (Wiga), *L. micdadei* (PI-1), *L. dumoffii* (NY-23), *L. gormanii* (LS-13), *L. longbeachae* serogroups 1 (Long Beach 4) and 2 (Tucker 1), and *L. jordanis* (BL-540) were obtained from the Centers for Disease Control, Atlanta, Ga., USA, and maintained by serial passage on buffered charcoal/yeast extract agar (Feeley *et al.*, 1979; Pasculle *et al.*, 1980).

Media. Buffered yeast extract (BYE) medium contained 1% (w/v) yeast extract, 1% (w/v) ACES, 0.040% (w/v) L-cysteine hydrochloride and 0.025% (w/v) soluble ferric pyrophosphate and was adjusted to pH 6.9 with potassium hydroxide (Pasculle *et al.*, 1980). Augmented buffered yeast extract (ABYE) medium was BYE supplemented with 0.5% (w/v) potassium 2-oxoglutarate and 1% (w/v) bovine serum albumin (Cohn's fraction V). Dialysed buffered yeast extract (DBYE) medium was BYE in which a dialysate of 10 g yeast extract per litre of medium was substituted for yeast extract powder. Solid media contained 1.7% (w/v) agar.

Haemolysis. Blood agar plates were prepared with 5% sterile defibrinated blood as previously described (Baine *et al.*, 1979a). Cultures were examined daily after inoculation, and the extent of haemolysis was graded semiquantitatively from – (absent) to + + + + (extensive).

Digestion of egg yolk and lecithin. Egg-yolk agar contained 5% (v/v) sterile hen's egg yolk as previously described (Baine *et al.*, 1979a). Lecithin agar contained L- α -phosphatidylcholine from fresh-frozen egg yolk (type IX-E, approximately 60% pure; Sigma), which was dispersed by vortexing the molten autoclaved medium just before it was dispensed into Petri dishes. Plates were incubated at 35 °C and examined daily after inoculation for the development of superficial iridescence of the culture, and for the appearance of zones of translucent clearing and an opaque precipitate within the medium, suggesting the activity of lipase, protease and lecithinase (phospholipase C), respectively (Allen & Siders, 1980). These changes were graded semiquantitatively from – (absent) to + + + + (extensive).

Hydrolysis of *p*-nitrophenylphosphorylcholine. Liberation of yellow *p*-nitrophenol from *p*-nitrophenylphosphorylcholine (Sigma), a water-soluble analogue of phosphatidylcholine, was used as a measure of phospholipase C activity (Kurioka & Matsuda, 1976). In a qualitative plate assay, seven species of *Legionella* were inoculated on DBYE agar containing 19.6 mM-*p*-nitrophenylphosphorylcholine, and the cultures were incubated at 37 °C.

For a quantitative assay of phospholipase C activity, 250 ml volumes of BYE broth in 1000 ml Erlenmeyer flasks were inoculated with *Legionella* strains, and the cultures were incubated for 2 d at 37 °C in a shaker bath with agitation at 180 r.p.m. After sedimentation (13 200 g, 10 min) the packed cells were washed 3 times in 5.0 mM-Tris (pH 7.2), 2.0 mM-CaCl₂, and resuspended in 1.5 ml volumes in the same buffer to give suspensions with a calculated optical density at 590 nm of 2.00 over a 1 cm light path (Bausch & Lomb type 33-29-40 colorimeter). Equal 1.5 ml volumes of the suspension of bacteria and of sterile 40 mM-*p*-nitrophenylphosphorylcholine in the same buffer were mixed in screw-cap tubes and placed in a shaker bath which was operated at 37 °C and 180 r.p.m. Control tubes containing bacteria without substrate, substrate without bacteria and buffer alone were processed in parallel. After overnight incubation, bacterial cells were sedimented by centrifugation, and hydrolysis of *p*-nitrophenylphosphorylcholine was assayed by measuring the absorbance of the supernates at 410 nm with correction for absorbance in control tubes. The concentration of the liberated chromophore was calculated with a programmable calculator from standard absorption curves prepared from solutions of *p*-nitrophenol at known concentrations.

The specificity of the method was assessed with commercially supplied phospholipases C and D. *C. perfringens* phospholipase C (type I, 8.1 U mg⁻¹), peanut phospholipase D (types II, 85 U mg⁻¹, and III, 155 U mg⁻¹), and cabbage phospholipase D (type IV, 90 U mg⁻¹) were obtained from Sigma. Solutions of these enzymes at final concentrations of 6.5 U ml⁻¹, 36 U ml⁻¹, 120 U ml⁻¹ and 81 U ml⁻¹, respectively, were incubated overnight at 37 °C in 200 μ l mixtures containing 20 mM-*p*-nitrophenylphosphorylcholine in 0.1 M-Tris (pH 7.65), 7.0 mM-CaCl₂, 3.0 mM-NaN₃. The absorbance of each mixture at 410 nm was measured after addition of 2.0 ml distilled water to each tube. The concentration of *p*-nitrophenol was determined as previously described and expressed as mmol liberated per unit of phospholipase C or phospholipase D activity, as appropriate.

Hydrolysis of tritiated lecithin. Phospholipase C activity in *Legionella* was assayed directly by a modification of the procedure of Grossman *et al.* (1974) using lecithin labelled with tritium in the methyl groups of the choline moiety. *Legionella* strains were grown in BYE broth and harvested as described above. Sedimented bacterial cells were washed three times in sterile distilled water and resuspended in distilled water to give suspensions with a calculated optical density of 15.0 at 590 nm over a 1 cm light path. L- α -Dipalmitoyl[*choline-methyl*-³H]phosphatidylcholine (specific activity 27 Ci mmol⁻¹; New England Nuclear) was dispersed in 0.1 M-Tris (pH 7.65), 3.5 mM-CaCl₂, 0.16% (v/v) Triton X-100, and sterilized by membrane filtration. Unlabelled L- α -dipalmitoylphosphatidylcholine (Sigma) was dispersed in 0.1 M-Tris (pH 7.65), 3.5 mM-CaCl₂, 0.16% (v/v) Triton X-100, sterilized by autoclaving, and stored at –20 °C; the unlabelled lecithin was sonicated (sonicator cell disruptor, model W185F, Heat Systems-Ultrasonics, Plainview, NY, USA) immediately before use to maximize dispersion of micelles. The final reaction mixtures contained 1.0 ml *Legionella* suspension or sterile distilled water, 0.1 μ Ci

tritiated lecithin ml^{-1} , 1.4 mM-unlabelled lecithin, 71 mM-Tris (pH 7.65), 2.5 mM- CaCl_2 , and 0.11% (v/v) Triton X-100 in a total volume of 3.5 ml in sterile screw-cap tubes.

After overnight incubation of the mixtures at 37 °C with agitation at 180 r.p.m. or at 4 °C without agitation, the bacterial cells were sedimented, and 2 ml volumes of the supernates were mixed with 0.57 ml 6.0% (v/v) HClO_4 and 1 ml distilled water. Portions (2 ml) of the acidified samples were serially extracted with 10 ml each of diethyl ether saturated with water, petroleum ether (boiling range 35–58 °C), and chloroform saturated with water to remove tritiated lecithin and choline (Gulewitsch, 1898; Renshaw, 1910), leaving phosphorylcholine in the acidified aqueous phase. Portions (1 ml) of the extracted aqueous phase from each sample were then dispensed to vials containing 10 ml liquid scintillation counting solution (Handifluor, Mallinckrodt, St Louis, Mo., USA), and the radioactivity present was determined by liquid scintillation counting (LS-330, LS-350 or LS-355 liquid scintillation system, Beckman Instruments). Radioactivity in the acidified aqueous phase of the samples containing distilled water blanks in place of *Legionella* suspensions was also determined without prior extraction with organic solvents. Phospholipase C activity was measured as the proportion of tritiated label released into the acidified aqueous phase as labelled phosphorylcholine as follows: [(c.p.m. in extracted *Legionella* sample) – (c.p.m. in extracted water blank)] $\times$ (c.p.m. in unextracted water blank) $^{-1}$.

The specificity of this assay for phospholipase C depends upon quantitative extraction of free choline as well as intact lecithin from the acidified aqueous phase, leaving only labelled phosphorylcholine to be counted. If choline were not reliably extracted, the assay could not distinguish between phospholipase C and phospholipase D, which hydrolyses lecithin at the bond between the phosphate group and the terminal choline moiety. The specificity of the method was verified with commercially supplied phospholipases C and D at the same concentrations used for validation of the colorimetric assay.

Enzyme solutions were incubated at 37 °C in a final volume of 200 μl in a tightly capped tube with 250 nCi L- α -dipalmitoyl[*choline-methyl*- ^3H]phosphatidylcholine ml^{-1} , 1.0 mM-unlabelled L- α -dipalmitoylphosphatidylcholine, 0.1 M-Tris (pH 7.65), 7.0 mM- CaCl_2 , 3.0 mM- NaN_3 , and 0.11% (v/v) Triton X-100. After overnight incubation the mixtures were brought to 2 ml with 0.11% (v/v) Triton X-100 in distilled water. The samples were then acidified, diluted and extracted and the tritium label in the aqueous phase was measured as described above. Radioactivity in the acidified aqueous phase of blank samples without added enzyme was also determined without prior extraction with organic solvents. Phospholipase C activity was measured as previously described and expressed as nmol tritiated phosphorylcholine released into the acidified aqueous phase per unit of phospholipase C or phospholipase D activity.

RESULTS

Haemolysis

Ten strains of *Legionella* comprising *L. pneumophila* (Philadelphia 1, Pontiac 1, Rockport 1, Dallas 1E, Chicago 2), *L. micdadei* (PI-1), *L. dumoffii* (NY-23), *L. longbeachae* (Long Beach 4, Tucker 2) and *L. jordanis* (BL-540) grew on ABYE agar with rabbit blood after overnight incubation at 35 °C. The cultures exhibited various degrees of partial or complete haemolysis, which was first apparent after 2 d incubation and became progressively more distinct over the ensuing 2 d. Haemolysis was most pronounced with the Dallas 1E strain of *L. pneumophila* and completely absent only with *L. micdadei*. Haemolysis was not enhanced by overnight refrigeration after 4 d at 35 °C nor by a second passage on blood agar.

Eight strains of *Legionella* were grown at 35 °C on ABYE agar with dog, guinea-pig, human, rabbit or sheep blood (Table 1). Dog red blood cells, with a high ratio of membrane phosphatidylcholine to sphingomyelin (Turner *et al.*, 1958), were most susceptible to lysis. Red blood cells of rabbit, guinea-pig and sheep showed intermediate degrees of susceptibility. Haemolysis was slight to absent on human blood agar. *L. micdadei* produced no haemolysis except on dog blood agar. The seven other *Legionella* strains were definitely haemolytic, with *L. pneumophila*, *L. bozemanii* and *L. longbeachae* exhibiting the strongest activity.

ABYE dog blood agar plates were inoculated with the same eight strains and incubated at various temperatures. No specific enhancement of haemolysis was observed at incubation temperatures above or below 35 °C. No major influence of the composition of the basal medium on haemolysis by *Legionella* was apparent when the same eight strains were grown on dog blood agar made with ABYE, BYE and F-G media (Feeley *et al.*, 1978). Divalent cations may variously enhance (Avigad, 1976) or diminish (Alouf, 1977) the activity of certain bacterial haemolysins. However, addition of 0.1 mM- or 1.0 mM- CaCl_2 or ZnCl_2 to BYE dog blood agar had no apparent effect on haemolysis by the same eight strains of *Legionella*; supplementation with 10 mM- CaCl_2 or ZnCl_2 inhibited bacterial growth.

Table 1. *Haemolysis of red blood cells from five mammalian species by Legionella spp.*

Cultures were incubated for 3 d on ABYE blood agar. The extent of haemolysis was graded from – (absent) to + + + + (extensive).

Species and strain	Red blood cell source:				
	Dog	Guinea-pig	Sheep	Rabbit	Human
<i>L. pneumophila</i> (Philadelphia 1)	+ + + + β^*	+	+	++	+
<i>L. pneumophila</i> (Dallas 1E)	+ + + + β^*	+ + +	++	+ + + +	+
<i>L. bozemanii</i> (Wiga)	+ + +	+ + +	+	+ + + +	+
<i>L. micdadei</i> (PI-1)	++	–	–	–	–
<i>L. dumoffii</i> (NY-23)	+ + + + β^*	+	+	++	$\pm$
<i>L. gormanii</i> (LS-13)	+ + + + β^*	+	+	++	$\pm$
<i>L. longbeachae</i> (Long Beach 4)	+ + +	++	++	+ + + +	+
<i>L. jordanis</i> (BL-540)	++	+	+	+	$\pm$

* Haemolysis progressed to yield a clear colourless zone around the bacterial growth, as with streptococcal β haemolysis.

Table 2. *Digestion of egg yolk by Legionella spp.*

Cultures were incubated for 7 d on ABYE egg-yolk agar. The changes were graded from – (absent) to + + + + (extensive).

Species and strain	Iridescence (Lipase)	Cleared zones (Protease)	Opaque precipitate (Lecithinase)
<i>L. pneumophila</i> (Philadelphia 1)	+ + +	+ + +	+
<i>L. bozemanii</i> (Wiga)	+ + +	+ + +	+ + +
<i>L. micdadei</i> (PI-1)	–	–	–
<i>L. dumoffii</i> (NY-23)	–	++	++
<i>L. gormanii</i> (LS-13)	+ + +	+ + +	++
<i>L. longbeachae</i> (Long Beach 4)	+ + +	+ + +	++
<i>L. jordanis</i> (BL-540)	+	+ + + +	+

Digestion of egg yolk and lecithin

Seven species of *Legionella* were grown at 35 °C on ABYE egg-yolk agar (Table 2). *L. micdadei* did not digest egg yolk. Apart from the absence of an iridescent sheen to the culture of *L. dumoffii*, presumptive lipase, protease and lecithinase reactions developed in the cultures of each of the other six species. *L. bozemanii* displayed the strongest presumptive lecithinase reaction.

Assessment of lecithinase activity on BYE agar incorporating approximately 1.8%, 0.60% or 0.47% (w/v) L- α -phosphatidylcholine was precluded by the inhibition of growth of these seven strains of *Legionella* on the lecithin-supplemented media. All seven species grew without formation of an opaque precipitate on BYE agar containing 0.49% (w/v) potassium 2-oxoglutarate and 0.29% (w/v) L- α -phosphatidylcholine, but this medium contained less than one-tenth the concentration of lecithin conventionally used to detect microbial phospholipases (Chrisope *et al.*, 1976).

Hydrolysis of p-nitrophenylphosphorylcholine

Within 2 d of inoculation of *L. bozemanii* on DBYE agar with *p*-nitrophenylphosphorylcholine, the medium developed a yellow cast, which progressively deepened over the ensuing 10 d. Fainter yellowing of the medium occurred with growth of *L. pneumophila* but not with cultures of *L. micdadei*, *L. dumoffii*, *L. gormanii*, *L. longbeachae* or *L. jordanis*.

Suspensions of washed cells of all the strains tested produced detectable hydrolysis of *p*-nitrophenylphosphorylcholine (Table 3). As in the qualitative plate assay, phospholipase C activity was greater with *L. bozemanii* than with other *Legionella* species. *C. perfringens* phospholipase C hydrolysed the chromogenic substrate; two of three vegetable phospholipase D preparations had lesser hydrolytic activity (Table 3).

Table 3. *Hydrolysis of p-nitrophenylphosphorylcholine by Legionella spp.*

Results are given as mean $\pm$ range of duplicate samples. Activity of *C. perfringens* phospholipase C: 6.29 ± 0.00 mmol *p*-nitrophenol liberated U^{-1} . Activities of reference phospholipases D: peanut, type II, 1.00 ± 0.00 mmol U^{-1} ; peanut, type III, 0.00 ± 0.00 mmol U^{-1} ; cabbage, 0.46 ± 0.00 mmol U^{-1} .

Species and strain	<i>p</i> -Nitrophenol liberated (μ mol per litre per OD ₅₉₀ unit of bacteria)
<i>L. pneumophila</i> (Philadelphia 1)	15.2 ± 1.3
<i>L. pneumophila</i> (Dallas 1E)	15.3 ± 0.3
<i>L. bozemanii</i> (Wiga)	247 ± 3
<i>L. micdadei</i> (PI-1)	
Expt 1	11.1 ± 0.5
Expt 2	7.4 ± 0.4
<i>L. dumoffii</i> (NY-23)	5.6 ± 0.5
<i>L. gormanii</i> (LS-13)	17.0 ± 0.0
<i>L. longbeachae</i> (Long Beach 4)	12.1 ± 0.5
<i>L. jordanis</i> (BL-540)	7.4 ± 0.3

Table 4. *Hydrolysis of L- α -dipalmitoyl[choline-methyl- 3H]phosphatidylcholine by Legionella spp. at 37 °C*

Results are given as mean $\pm$ range of duplicate samples. Activity of *C. perfringens* phospholipase C: 41.8 ± 1.0 nmol [3H]choline phosphate liberated U^{-1} . Activities of reference phospholipases D: peanut, type II, -0.3 ± 0.1 nmol U^{-1} ; peanut, type III, 0.2 ± 0.0 nmol U^{-1} ; cabbage, 1.2 ± 0.2 nmol U^{-1} .

Species and strain	Percentage of substrate hydrolysed
<i>L. pneumophila</i> (Philadelphia 1)	
Expt 1	99.1 ± 2.0
Expt 2	98.6 ± 5.0
<i>L. bozemanii</i> (Wiga)	
Expt 1	14.5 ± 5.2
Expt 2	2.8 ± 0.5
<i>L. micdadei</i> (PI-1)	0.1 ± 0.1
<i>L. dumoffii</i> (NY-23)	52.7 ± 6.9
<i>L. longbeachae</i> (Long Beach 4)	4.1 ± 0.2
<i>L. jordanis</i> (BL-540)	51.7 ± 5.3

Hydrolysis of tritiated lecithin

Extraction of label from the supernates of blanks containing no bacterial cells was 98.4–98.8% complete, indicating that the organic solvents effectively removed unmetabolized tritiated lecithin from the samples. Five of six *Legionella* species tested showed definite phospholipase C activity at 37 °C (Table 4). Enzyme activity was markedly inhibited at 4 °C (data not shown). Only *L. micdadei*, which also exhibited minimal haemolysis (Table 1) and did not digest egg yolk (Table 2), gave no significant hydrolysis of labelled lecithin (Table 4). The specificity of the assay with tritiated lecithin was confirmed by the results obtained with *C. perfringens* phospholipase C and phospholipases D of vegetable origin (Table 4).

DISCUSSION

An intense outpouring of inflammatory cells into affected alveoli characterizes pneumonia caused by *L. pneumophila*. Cytoclasis with necrosis of mononuclear and polymorphonuclear cells in the inflammatory exudate occurs (Winn & Myerowitz, 1981). This necrotizing process often yields small abscesses (Blackmon *et al.*, 1981; Winn & Myerowitz, 1981) and sometimes progresses to large cavities which are visible by roentgenography (Edwards & Finlayson, 1980; Lewin *et al.*, 1979; Magnusson & Israel, 1980).

Toxins produced *in vivo* by *Legionella* might damage alveolar cells or leucocytes in inflammatory infiltrates. An extracellular product of low molecular weight that is toxic to Chinese hamster ovary cells (Friedman *et al.*, 1980) and that disrupts the oxidative metabolism of polymorphonuclear leucocytes (Friedman *et al.*, 1982) has been isolated from *L. pneumophila*, but this substance apparently lacks cytolytic activity. *L. pneumophila* elaborates various proteases *in vitro* (Feeley *et al.*, 1978; Baine *et al.*, 1979*a*; Müller, 1981; Thompson *et al.*, 1981; Thorpe & Miller, 1981; Nolte *et al.*, 1982; Berdal *et al.*, 1983), but these enzymes are not likely to produce the leucocytoclasia and abscess formation seen in lungs infected with *Legionella*. A better candidate for a hypothetical product of *Legionella* organisms causing necrosis of inflammatory cells and pulmonary parenchyma would be a toxin acting on host cell membranes (Alouf, 1977).

The pathological effects of bacterial cytolysins are influenced by the anatomic site of infection. For example, profound intravascular haemolysis is produced by α -toxin in *Clostridium perfringens* septicaemia (MacLennan, 1962), whereas *C. perfringens* pneumonia produces necrosis of lung tissue without haemolysis (Bayer *et al.*, 1975). Erythrocytes are convenient target cells for the assay of bacterial cytolysins regardless of whether naturally acquired infections with a given pathogen cause intravascular infection and haemolysis.

Cultures of *L. pneumophila* show haemolytic activity (Baine *et al.*, 1979*a*), and urine and plasma from rabbits experimentally infected with *L. pneumophila* lyse guinea-pig red blood cells *in vitro* (Baine *et al.*, 1979*b*). Haemolytic activity has also been reported in supernates of cultures of *L. pneumophila* (Thorpe & Miller, 1984) and *L. bozemanii* (Frisch & Baine, 1984). This study demonstrates that cytolytic activity which is demonstrable *in vitro* with red blood cell targets is widespread in the genus *Legionella*.

Haemolytic cytolysins for which the mechanism of action is defined include surface-active agents, cholesterol-binding compounds, and phospholipases and related enzymes that hydrolyse phosphoglycerides and sphingomyelin (Arbuthnott, 1982). The haemolytic activity of some bacterial phospholipases C is well known (Möllby, 1978). Haemolysis induced by phospholipase C may be mediated by an increase in osmotic fragility resulting from enzymic modification of the red blood cell membrane (Taguchi *et al.*, 1983).

The predominant lipids of mammalian red blood cells are cholesterol, phosphatidylethanolamine, phosphatidylcholine (lecithin) and sphingomyelin. Marked interspecies differences exist in the ratio of the latter two phospholipids (Lehninger, 1975). Differences in the ratio of phosphatidylcholine to sphingomyelin may account for differences in the susceptibility of red blood cells from various mammalian species to lysis by a given bacterial phospholipid-hydrolysing toxin (Liu, 1977). Red blood cells from guinea-pigs have previously been found to be more susceptible to haemolysis by six strains of *L. pneumophila* than those from rabbits, sheep, horses or humans (Baine *et al.*, 1979*a*). In the present study, red blood cells from dogs were lysed by *Legionella* strains even more readily than those from guinea-pigs. The red blood cell membranes in both dogs and guinea-pigs contain a high ratio of phosphatidylcholine to sphingomyelin. Sheep red blood cells, with a low ratio of membrane phosphatidylcholine to sphingomyelin (Turner *et al.*, 1958), were only moderately susceptible to lysis by *Legionella*. However, the phospholipid composition of the red blood cell membrane is not the sole determinant of susceptibility to haemolysis by *Legionella* strains. Membranes of rabbit and human red blood cells are similar in phospholipid content (Turner *et al.*, 1958), but there were marked differences in *Legionella* haemolytic activity against cells from these two species.

Phosphatidylcholine is not found in most prokaryotes but it is the most abundant phospholipid in cells of *L. pneumophila* (Finnerty *et al.*, 1979). It would not therefore be surprising if *Legionella* species could produce enzymes that metabolize this substance. Cultures of *L. pneumophila* on agar with 5% egg yolk have previously been reported to produce clouding of the medium similar to that caused by lecithinase-positive species of *Clostridium* (Baine *et al.*, 1979*a*). This observation has been confirmed by Thorpe & Miller (1981) but they found no evidence of lecithinase in cultures on agar containing 3% L- α -phosphatidylcholine. However, concentrations of L- α -phosphatidylcholine that are sufficient to permit assessment of lecithinase activity in plate assays appear to inhibit growth of *Legionella*.

The presumptive lecithinase activity shown on egg-yolk agar by six of the seven *Legionella* species tested was confirmed by more specific assays of bacterial cell suspensions with *p*-nitrophenylphosphorylcholine and tritiated lecithin. Apparent discrepancies between activities using the chromogenic and radiolabelled substrates may imply differences in enzyme specificity for the diacylglycerol moiety of phosphatidylcholine. For example, the synthetic phosphatide *p*-nitrophenylphosphorylcholine can be regarded equally well as an analogue of phosphatidylcholine or of sphingomyelin. Variation in the extent of hydrolysis of tritiated lecithin between experiments with a given species and between experiments with different species may also have been influenced by incubation of the cells at concentrations of Triton X-100 that were close to those that in preliminary experiments were found to kill *Legionella* cells.

L. micdadei, which is genetically distant from other species of *Legionella* (Hébert *et al.*, 1980), was atypical in its minimal haemolytic activity and failure to digest egg yolk and to hydrolyse tritiated lecithin. No definite association between relative haemolytic potency and apparent phospholipase C activity was apparent among the other *Legionella* species examined. This finding may reflect the merely semiquantitative assessment of haemolytic activity but it is also compatible with mediation of haemolysis by other substances in addition to, or instead of phospholipase C in at least some species of *Legionella*.

Intrapulmonary liberation of phospholipase C might not only damage host cell membranes but also compromise pulmonary compliance by hydrolysis of dipalmitoylphosphatidylcholine (Klaus *et al.*, 1961), the chief constituent of lung surfactant (Scarpelli, 1968). Alveolar hyaline membrane formation has been reported in legionellosis (Winn & Myerowitz, 1981), raising the question of whether damage to the surfactant layer occurs.

Lung tissue infected with *L. pneumophila* contains numerous bacteria within alveolar macrophages (Blackmon *et al.*, 1981; Winn & Myerowitz, 1981). Horwitz and colleagues have investigated the interaction between phagocytes and this pathogen. Like *Listeria monocytogenes* and *Mycobacterium tuberculosis*, *L. pneumophila* is a facultatively intracellular parasite, multiplying *in vitro* in unstimulated human monocytes (Horwitz & Silverstein, 1980, 1981) and macrophages (Nash *et al.*, 1982) under conditions that do not permit extracellular replication. Intracellular multiplication within human monocytes is associated with the failure of lysosomes to fuse with phagosomes laden with live *Legionella*, but phagosome-lysosome fusion is not inhibited in monocytes ingesting killed bacteria (Horwitz, 1983). Thus live *L. pneumophila* cells may produce a factor perturbing the phagosome membrane so that the phagocytic vacuole is no longer receptive to fusion with lysosomes.

Diacylglycerol, formed in the hydrolysis of phosphatidylcholine by phospholipase C, causes perturbation of the membranes of erythrocytes and mast cells (Allan *et al.*, 1975, 1976; Kennerly *et al.*, 1979; Sullivan, 1981). Ahkong *et al.* (1973) have shown that application of glyceryl dioleate to the exterior of hen erythrocytes suspended in buffer induces them to fuse. The consequences of elevating the diacylglycerol concentration in the interior of an intramonocytic phagosome are not known, but the question arises of whether perturbation of the vacuole membrane by intraphagosomal release of phospholipase C by *Legionella* organisms could interfere with phagosome-lysosome fusion. Phospholipase C from *C. perfringens* has also been shown to inhibit chemotactic migration of human monocytes (Wilkinson, 1975), possibly by reducing binding of chemotactic substances to the phagocytes (Wilkinson & Allan, 1978).

Although this study does not establish that haemolytic species of *Legionella* necessarily produce extracellular haemolysins, progress in the purification of haemolytic cytolysins from culture supernates of *L. pneumophila* (Thorpe & Miller, 1984) and *L. bozemanii* (Frisch & Baine, 1984) has already been reported. Further work is necessary to establish the optimal conditions for production of cytolysins and phospholipases C by various species of *Legionella*, to purify and characterize these products, and to evaluate the contribution of phospholipase C activity to haemolytic potency in each species. To assess the significance of haemolytic cytolysins and phospholipases C in the pathogenesis of legionellosis, it will be useful to generate mutants deficient in one or both activities for comparison with wild-type strains in laboratory models of virulence and phagocytosis.

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