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Opsonization of *Legionella pneumophila* in human serum: key roles for specific antibodies and the classical complement pathway

H. A. VERBRUGH,* D. A. LEE,* G. R. ELLIOTT,* W. F. KEANE,† J. R. HOIDAL† & P. K. PETERSON† *The Departments of *Pediatrics and †Medicine, University of Minnesota School of Medicine, Minneapolis, Minnesota, U.S.A.*

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Summary. *Legionella pneumophila* has previously been shown to require serum factors for efficient uptake by phagocytic cells. In this investigation, the roles of specific antibody and complement in phagocytosis of *L. pneumophila* by human polymorphonuclear leucocytes (PMN) and tissue macrophages were determined. Opsonization was assessed by quantitating the uptake of [^{3}H]-labelled *Legionellae*. Compared to other Gram-negative and to Gram-positive bacterial species, *L. pneumophila* was highly resistant to the opsonic activity of normal pooled human serum (PHS). Of 12 donor sera tested, only four promoted significant *L. pneumophila* uptake when used at full strength. Experiments with immune antibody, and with human sera deficient in immunoglobulins, or the complement components C2, C3, or C5, revealed that *L. pneumophila* opsonization was dependent on antibody-mediated activation of the classical complement pathway; activation of the alternative pathway could not be detected. At high concentrations, immune antibody alone could adequately opsonize *L. pneumophila*. Human alveolar and peritoneal macrophages required very similar amounts and types of opsonins for *L. pneumophila* phagocytosis as did human PMN. Heating *L. pneumophila* to temperatures $\geq 80°$ abolished its resistance to opsonization by diluted PHS; however, activation of complement via the alternative pathway or via other antibody-independent routes remained undetectable. These studies show that, in addition to immune antibody, the classical pathway of complement plays an important role in the opsonization of *L. pneumophila*. The limited ability of these bacteria to interact with human complement provides a likely explanation for their resistance to opsonization and may be partly based on heat-sensitive structures on the surface of *L. pneumophila*.

Correspondence and present address: Dr Henri A. Verbrugh, Laboratory for Microbiology, Catharijnesingel 59, 3511 GG Utrecht, The Netherlands.

INTRODUCTION

The Legionnaires' disease bacterium, *Legionella pneumophila*, is capable of intracellular growth in human fibroblasts and in mononuclear phagocytes (Wong *et al.*, 1980; Kishimoto *et al.*, 1979; Horwitz & Silverstein, 1980b). The mechanism underlying the intracellular multiplication of *L. pneumophila* in monocytes was recently clarified by the demonstration of an inhibition in the fusion between phagosomes and lysosomes in the cytoplasm of these phagocytes (Horwitz, 1983). Much less is known, however, about the initial steps in the interaction of *L. pneumophila* and phagocytes, i.e. the attachment and ingestion phase of the bacteria-phagocyte encounter. It has previously been noted that efficient uptake of *L. pneumophila* by phagocytes required the presence of both specific antibody and complement (Horwitz & Silverstein, 1980a). However, successful *in vitro* infection of cells

has been accomplished in media containing heat-inactivated fetal calf serum as the only potential source of opsonins (Wong *et al.*, 1980; Daisy *et al.*, 1981). On the other hand, Johnson, Pesanti & Elliott (1979) demonstrated a 7–11-fold increase in the uptake of *L. pneumophila* by mouse peritoneal macrophages when serogroup-specific rabbit antiserum was added to their incubation mixtures.

In order to define further the opsonic requirements of these bacteria, we studied the uptake of the Philadelphia 1 strain of *L. pneumophila* by human polymorphonuclear and mononuclear phagocytes in an *in vitro* assay which uses radiolabelled bacteria. The results suggest that *L. pneumophila* is relatively resistant to the opsonic activity of normal, non-immune human serum and requires specific antibody for optimal phagocytosis. The complement system can contribute greatly to *L. pneumophila* opsonization, but its activity was found to be restricted to classical pathway activation via antibody. Surprisingly, *L. pneumophila* did not activate complement via the alternative pathway.

MATERIALS AND METHODS

Organisms and cultural conditions

The Philadelphia 1 strain of *L. pneumophila* was obtained from the Centers for Disease Control (CDC, Atlanta, GA) as a frozen spleen suspension. In order to assure bacterial viability and virulence, *L. pneumophila* was passed once in embryonated hen eggs. Yolk sacs were harvested from those eggs whose embryos died after 3 days. After testing for viability and for the absence of contaminating bacteria, a stock suspension was prepared and stored at −70°. For each experiment, a small amount of frozen stock suspension was thawed in 1 ml of sterile phosphate-buffered saline, pH 7·4 (PHS), and then plated onto buffered charcoal yeast extract (BCYE) agar containing 20 μCi [^{3}H]thymidine (specific activity, 50 μCi/mmol, ICN, Irvine, CA). After 3–4 days' growth at 37°, *L. pneumophila* colonies were taken from the agar surface with a sterile moistened swab, suspended in sterile PBS and washed twice with PBS. A final suspension of $0{\cdot}5–1{\cdot}0 \times 10^9$ CFU/ml was made in Hanks' balanced salt solution containing 0·1% gelatin (GHBSS). In some experiments, *L. pneumophila* suspensions were held for 1 hr at indicated temperatures prior to use in the opsonization procedure. Also used in this study were the encapsulated Smith strain of *Staphylococcus aureus* and its unencapsulated variant strain (Peterson *et al.*, 1978), two encapsulated clinical isolates of *Escherichia coli* (serotype 075: K1 and serotype 018:K?) (van Dijk *et al.*, 1979), one unencapsulated strain of *E. coli* (serotype 022: H16: K-) (Verbrugh *et al.*, 1982a), and a capsular type VII strain of *Streptococcus pneumoniae* (kindly donated by Dr G. S. Giebink, Minneapolis, MN) (Giebink *et al.*, 1980). These bacteria were grown in 10 ml Mueller-Hinton broth (Difco Laboratories, Detroit, MI) to which 20 μCi of (2,8[^{3}H]) adenine (ICN) or (methyl[^{3}H]) thymidine (ICN) was added, as previously described (Verbrugh *et al.*, 1982a; Giebink *et al.*, 1980). After 18 hr incubation at 37°, bacterial suspensions in GHBSS were prepared as described for *L. pneumophila* (*vide supra*).

Opsonic sources

Serum was collected from 12 healthy donors who denied a history of Legionnaires' disease. The sera were stored individually and as a serum pool at −70°. Serum was also obtained from patients with genetically determined complete and selective deficiencies of complement factors C2 (Kim *et al.*, 1977), C3 (Roord *et al.*, 1982) or C5 (Verbrugh *et al.*, 1982b).

Immunoglobulin-deficient serum was from a male patient with primary X-linked agammaglobulinaemia (Bruton type) and contained less than 5 mg/dl of IgG and undetectable levels of IgM, IgA, IgE and IgD. Heated serum was incubated at 56° for 30 min. In some experiments, serum was chelated with 10 mM ethyleneglycoltetraacetic acid (EGTA, Sigma Chemical Co., St Louis, MO) in the presence of 5 mM $MgCl_2$ (MgEGTA). Immune rabbit IgG specific for *L. pneumophila* serogroup I was kindly donated by R. Benson (CDC). Just prior to use, the opsonic mixture were constituted using GHBSS as diluent.

Opsonization procedure

One-hundred ml of the bacterial suspension was mixed with 0·9 ml of opsonin and incubated at 37° for 30 min. The suspensions were then centrifuged (15 min at 2000 *g*), and the bacterial pellets were resuspended in 1·0 ml of GHBSS and held at 4° until use (preopsonized bacteria).

Quantitation of bacterial C3 fixation

The amount of C3 that became fixed to the surface of *Legionellae* during opsonization was determined by quantitative fluorescent immunoassay as previously described (Verbrugh *et al.*, 1979). In this assay, preopsonized bacteria are washed three times with

PBS, reacted with fluorescein-conjugated goat IgG monospecific for human C3 (Cappel Lab., Cochranville, PA; Lot no. 14724), and then washed again with PBS.

The final bacterial pellets were resuspended with 2·5 ml 0·1 M NaOH which will dissociate the conjugate. The intensity of the fluorescence of the supernatant was then measured in a Perkin-Elmer 204 Fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT) with exitation and emission wavelengths of 485 and 525 nm, respectively. The results are given as percentages of C3 fixation ranging from zero (control using non-opsonized bacteria) to 100% (maximal emission intensity in the series tested), which directly correlates with the amounts of C3 bound to the bacteria (Verbrugh *et al.*, 1979).

Polymorphonuclear leucocytes

Suspensions of pure PMN were prepared from heparinized venous blood of healthy donors, as previously described (Verbrugh *et al.*, 1979). Final cell suspensions contained 5×10^6 PMN/ml GHBSS. Purity of the PMN suspensions and viability by trypan blue exclusion both exceeded 95%.

Mononuclear phagocytes

Alveolar macrophages (AMØ) were obtained from normal donors by subsegmental saline lavage of the lingula of the left lung, or the middle lobe of the right lung, as previously described (Hoidal, White & Repine, 1979). AMØ in the recovered lavage fluid were washed three times with GHBSS and resuspended at a concentration of 5×10^6 AMØ/ml GHBSS. Purity of AMØ suspension was $\geqq 85\%$ (remaining cells were primarily lymphocytes) and viability exceeded 90%.

Human peritoneal macrophages (PMØ) were isolated from peritoneal effluents of uninfected patients undergoing maintenance peritoneal dialysis for end-stage renal disease, essentially as previously described (Verbrugh *et al.*, 1983). PMØ were harvested from 3 litres of effluent by centrifugation (1,600 *g* for 30 min), washed and resuspended to a final concentration of 5×10^6 PMØ/ml of GHBSS. The final cell suspension contained a mean of 86% PMØ (range, 78–91%), 8% lymphocytes (range 3–15%), 4% PMN (range 0–9%), and 2% eosinophils (range 0–6%). More than 95% of the PMØ were viable by trypan blue exclusion.

Measurement of bacterial opsonization

Bacterial opsonization was assessed in a quantitative phagocytosis assay which determines the uptake of radiolabelled bacteria by human PMN, AMØ and PMØ. The assay has been described in detail previously (Peterson *et al.*, 1977). Briefly, 0·1 ml of preopsonized radiolabelled bacteria (5×10^6 CFU) was mixed with 0·1 ml of phagocytic cell suspension (5×10^5 cells), and phagocytosis was allowed to proceed for 30 min in a shaking incubator at 37°. Different lengths of incubations were also used when indicated. Phagocytosis was stopped by adding ice-cold PBS, and non-leucocyte-associated bacteria were removed by three cycles of differential centrifugation. Phagocyte-associated radioactivity was determined in a liquid scintillation counter, and has been shown to accurately reflect the degree of phagocytosis (Peterson *et al.*, 1977). Phagocytosis was expressed as a percentage uptake of the total added radioactivity and was taken as a measure of bacterial opsonization (Verbrugh *et al.*, 1982b).

Measurement of complement consumption

Bacterial samples of 0·1 ml, at specified concentrations and of PBS (control), were added to polypropylene vials containing 1·0 ml of the indicated serum source. After incubation for 60 min in the incubator shaker (250 r.p.m., 37°), the vials were centrifuged at 2000 *g* for 15 min (4°), and the supernatants were collected for measurement of residual C3–9 haemolytic activity. The assay for C3–9 haemolytic activity was carried out as previously reported (Kim *et al.*, 1977). The haemolytic C3–9 activity remaining in the serum samples was compared to that of serum incubated with PBS alone and results were expressed as percent C3–9 consumed.

Electron microscopy of phagocytic mixtures

In order to obtain electron micrographs of *L. pneumophila* and of phagocytic cells, suspensions were prepared for transmission electron microscopy as previously described (Clawson, 1973) and viewed in an electron microscope (model 201; Philips Electronic Instruments, Eindhoven, The Netherlands).

RESULTS

L. pneumophila resistance to opsonization

In order to evaluate the relative resistance of *L. pneumophila* to the opsonic activity of normal non-immune human serum, phagocytosis of *L. pneumophila* was compared to that of other bacterial species following opsonization in various dilutions of pooled human serum (Table 1). *L. pneumophila* proved to be

Table 1. Relative resistance of *Legionella pneumophila* to opsonization with pooled normal human serum

Organism	Phagocytosis (% uptake*) by PMN after opsonization† in serum at a concentration of:				
	100%	50%	10%	1%	0·1%
L. pneumophila (serogroup 1)	34	13	8	7	5
E. coli (022:H16)	50	52	52	23	20
E. coli (075:K1)	39	38	10	7	9
E. coli (018:K?)	43	40	19	9	5
S. aureus (encapsulated)	72	70	35	12	9
S. aureus (unencapsulated)	62	60	60	45	41
S. pneumoniae (type VII)	68	67	56	4	2

* Uptake of preopsonized bacteria by PMN was determined after 30 min incubation at 37°. The bacteria to PMN ratio was approximately 10:1.

† Bacteria, 0·1 ml (5×10^7 CFU), were incubated in pooled human serum, 0·9 ml, at indicated concentrations for 30 min at 37°, centrifuged and resuspended in 1·0 ml GHBSS.

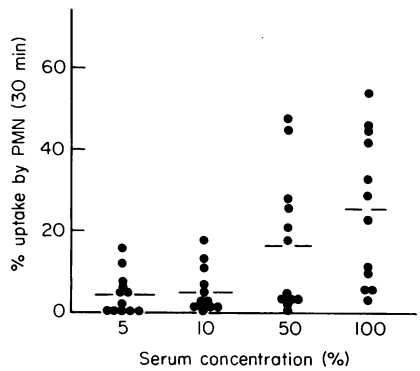

Figure 1. *L. pneumophila* opsonization in serum from 12 healthy donors. Bacteria were preopsonized for 30 min in serum from healthy individuals without a history of Legionnaires' disease at indicated concentrations. Uptake by PMN was determined after 30 min.

more resistant to opsonization by normal human serum than other bacterial strains previously noted to be poorly opsonized in normal serum, including encapsulated *E. coli* and *S. aureus*. PMN uptake of *L. pneumophilia* was consistently found only after opsonization in undiluted pooled serum. The uptake of *L. pneumophila* was poor after opsonization in serum diluted 1:2, and virtually no uptake could be detected when lower concentrations of serum were used (Table 1). The capacity of 12 individual donor sera to opsonize *L. pneumophila* is shown in Fig. 1. Interestingly, a considerable variation was found in the *L. pneumophila* opsonic activity of donor sera. In five sera, essentially no opsonic activity of *L. pneumophila* could be detected; these sera did not promote *L. pneumophila* phagocytosis even when used undiluted. In one serum, low level opsonic activity, i.e. 23% uptake, was present when used undiluted. The remaining six sera promoted low level *L. pneumophila* uptake at 50% concentration. Adequate opsonic activity, i.e. greater than 40% uptake, was present in only two (17%) sera when used at a 50% concentration; however, no serum was adequately opsonic at lower concentration (Fig. 1). In order to investigate whether *L. pneumophila* opsonic activity is a stable characteristic, serum was collected from donors at several intervals over a 6-month period. Titration experiments revealed that *L. pneumophila* opsonic activity, when detectable in the serum of a healthy donor, remains relatively stable over time (data not shown). Furthermore, opsonic activity remained undetectable in sera from two donors that initially did not promote the uptake of *L. pneumophila* (data not shown).

Role of antibody and complement in *L. pneumophila* opsonization

Specific antibody and the heat-labile complement system are the major sources of opsonins in human serum. In order to delineate the role of these serum factors in the opsonization of *L. pneumophila* titration more clearly, experiments were performed using pooled serum, pooled serum from which complement activity was removed by heat treatment, agammaglobulinaemic serum, and solutions containing increasing amounts of *L. pneumophila* immune antibody with or without complement. The results of these experiments, summarized in Fig. 3, indicate that both antibody and complement may be important for *L. pneumophila* opsonization. Thus, the opsonic activity of 50% and 100% pooled serum proved to be largely heat-labile. In the absence of immunoglobulins (agammaglobulinaemic serum), however, *L. pneumophila* was not opsonized, indicating a need for antibody (Fig. 2). This opsonic activity of antibody was exemplified further by the capacity of immune IgG at concentrations $\geqq 1$ μg/ml to adequately promote *L. pneumophila* phagocytosis in the absence of other serum factors. However, the addition of fresh serum as a source of complement greatly enhanced the opsonic activity of low level immune antibody. Ten percent pooled serum

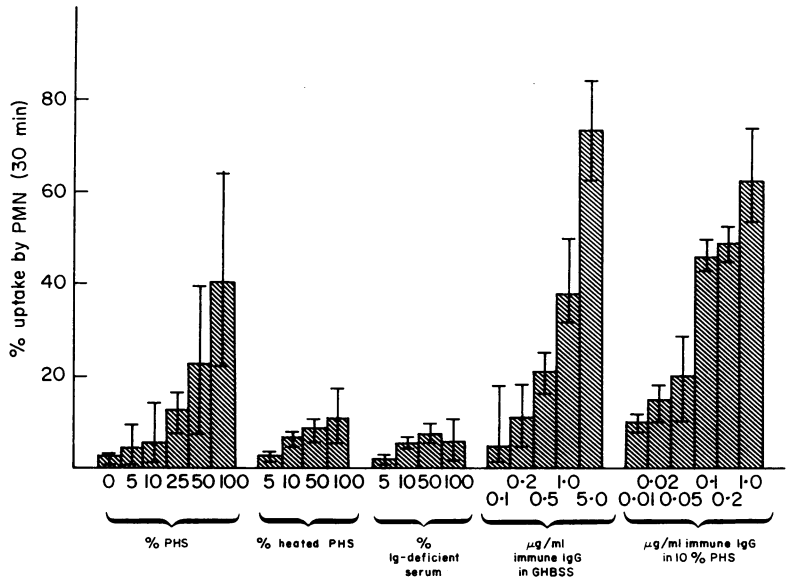

Figure 2. Role of complement and immune antibody in opsonization of *L. pneumophila*. *L. pneumophila* was opsonized in indicated concentrations of either human serum (PHS), in heated PHS, or immunoglobulin (IG)-deficient serum from a patient with X-linked agammaglobulinaemia; likewise, bacteria were preopsonized in solutions containing increasing amounts of immune IgG with either buffer (GHBSS) or 10% PHS in GHBSS as diluent. After opsonization, bacteria were washed and mixed with normal PMN. Uptake was recorded after 30 min incubation. Bars indicate means and parentheses the total range based on at least three separate experiments.

combined with 0·1 μg/ml or 0·2 μg/ml immune antibody was opsonic for *L. pneumophila*, whereas 10% serum alone or these low levels of antibody alone were not opsonic (Fig. 2).

Complement components involved in *L. pneumophila* opsonization

The activation of the third component of complement (C3) with the deposition of C3b on the bacterial surface is known to be the crucial step in complement-mediated opsonization and phagocytosis by PMN (Stossel *et al.*, 1975). C3 can become firmly attached to the surface of bacteria by activation of either the classical or the alternative complement pathway. In order to establish the role of C3 and its route(s) of activation in *L. pneumophila* opsonization, we used human sera with genetically determined deficiencies of complement components C2, C3 or C5. None of these sera promoted *L. pneumophila* phagocytosis without the addition of immune antibody. When low levels of *L. pneumophila* immune antibody were added, however, phagocytosis of *L. pneumophila* preopsonized in C5-deficient serum or in normal pooled serum was demonstrated (Fig 3). In sharp contrast, no uptake was seen of *L. pneumophila* opsonized in either C2-deficient or C3-deficient serum (Fig. 3). *L. pneumophila* opsonization thus appeared to be dependent on the presence of C3 and an intact classical complement pathway. Activation of C5 and the later components of complement were not required.

Lack of alternative pathway activation by *L. pneumophila*

Since *L. pneumophila* could not be opsonized in human C2-deficient serum, an inability of *L. pneumophila* to activate the alternative complement pathway was postulated. To test this hypothesis, we determined the ability of *L. pneumophila* to activate and consume haemolytic C3–9 in normal human serum and in human serum which was chelated with MgEGTA to block activation of the classicial pathway of complement. Compared to an unencapsulated *E. coli* strain, *L. pneumophila* consumed virtually no C3–9 in normal serum or MgEGTA-chelated serum (Table 2). However, after the addition of immune antibody, C3–9 consumption was clearly present in normal serum, but

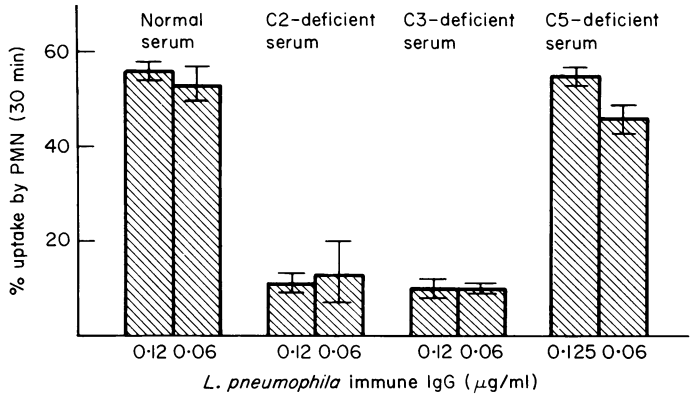

Figure 3. *L. pneumophila* opsonization mediated via antibody-dependent activation of the classical complement pathway. *L. pneumophila* was incubated (30 min) in normal human serum and in human sera with indicated complete deficiencies of individual complement components. Sera were used at a concentration of 25% with subopsonic concentrations of immune antibody added. After opsonization, bacteria were washed and mixed with normal PMN. Uptake was recorded after 30 min. Bars represent means and parentheses the total ranges of two separate experiments done in duplicate.

Table 2. Consumption of haemolytic C3–9 by *Legionella pneumophila*

Activating organism	Percent C3–9 consumption* in: PHS†	PHS with immune IgG‡	MgEGTA-PHS§	MgEGTA-PHS with immune IgG
L. pneumophila				
2×10^8 CFU/ml	1	83	0	0
1×10^8 CFU/ml	2	56	0	0
2×10^7 CFU/ml	1	36	3	0
E. coli (022:H16)				
2×10^8 CFU/ml	86	ND¶	87	ND
1×10^8 CFU/ml	58	ND	63	ND
2×10^7 CFU/ml	34	ND	40	ND

* Bacteria were incubated with sera at indicated final concentrations for 60 min at 37°. The haemolytic C3–9 activity remaining in the sera was then determined and compared to that of control serum incubated with buffer alone. Results are expressed as percent C3–9 consumed and are from one representative experiment.

† PHS, pooled human serum used at a 1/2 dilution.

‡ One mg *L. pneumophila* immune IgG per ml serum was added.

§ MgEGTA is PHS-chelated with 10 mM EGTA in the presence of 5 mM $MgCl_2$.

¶ ND, not done.

L. pneumophila remained unable to activate and consume C3–9 via the alternative complement pathway (Table 2). Thus, interactions of *L. pneumophila* (serogroup 1) with the human complement system are restricted to the classical pathway and depend upon the presence of immune antibody for optimal activation.

Uptake of *L. pneumophila* by human phagocytes of different origin

The phagocytosis experiments with preopsonized *L. pneumophila* were thus far carried out using human peripheral blood PMN as an easily obtainable source of phagocytic cells. In order to confirm true bacterial

uptake, i.e. ingestion, of *L. pneumophila* by those cells, we obtained electron micrographs of PMN/*L. pneumophila* mixtures at the end of the 30 min incubation time. The micrographs consistently showed human PMN with multiple intracellular vacuoles containing bacteria and contents of fused lysosomes, indicating phagocytosis of *L. pneumophila* by these cells (micrographs not shown). However, mononuclear phagocytes such as those lining the bronchoalveolar spaces, rather than blood PMN, may be more important in the initial phases of Legionnaires' disease. We therefore compared PMN, AMØ and PMØ as to their ability to phagocytize *L. pneumophila*. *L. pneumophila* was preopsonized in the presence of an excess of complement and antibody, as well as under more limiting opsonic conditions. In order to characterize the recognition process by these phagocytes more fully, the kinetics of *L. pneumophila* uptake were determined. The results presented in Fig. 4 demonstrate that the opsonic requirements for phagocytosis of *L. pneumophila* by either human PMN, AMØ or PMØ are very similar. Only minor differences were found in the kinetics of uptake of *L. pneumophila* by the different types of phagocytic cells. In addition, electron microscopy indicated that *L. pneumophila* was truly ingested by both AMØ and PMØ as was found with PMN (data not shown).

Opsonization of heat-treated *L. pneumophila*

In an effort to modify the outer surface of the Legionnaires' disease bacterium, and thereby potentially change its requirement for opsonins, it was found that prior heating of *L. pneumophila* significantly increased its sensitivity to opsonization by normal human serum. This change in opsonic requirements was temperature dependent. Thus, heating *L. pneumophila* at 80° or 100° for 1 hr made the strain increasingly susceptible to opsonization by 10% pooled human serum; temperatures of 56° or lower, however, were not effective in this regard (data not shown). By electron microscopy, heat-treated (100°) *L. pneumophila* appeared superficially intact, albeit that the granular and vacuolated pattern of the cytoplasm had changed significantly (Fig. 5). Further studies revealed that, although opsonization of heated *L. pneumophila* in 10% serum was complement-dependent, the bacteria remained unable to become opsonized in the absence of antibody or an intact classical complement pathway (Fig. 6). On the other hand, the uptake of *L. pneumophila* opsonized in immune antibody alone was only slightly improved by heating the bacteria (Fig. 6). Since the enhanced susceptibility to opsonization in normal serum could possibly be due to an increased deposition of opsoni-

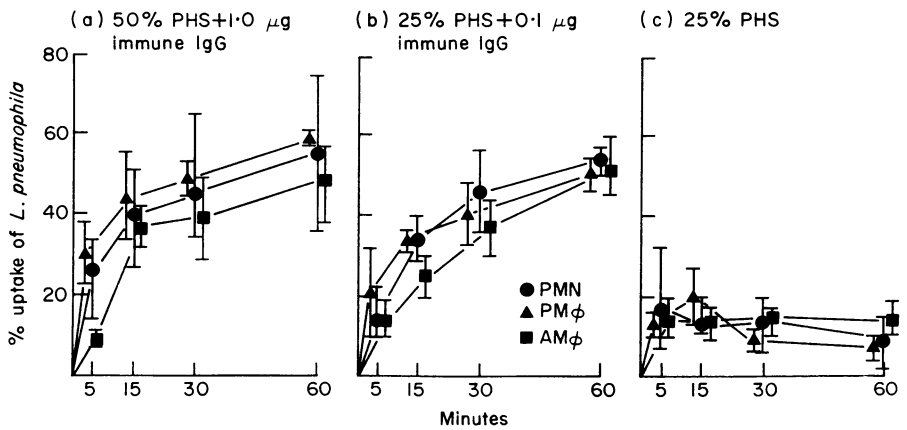

Figure 4.–Kinetics of *L. pneumophila* phagocytosis by human polymorphonuclear leucocytes (PMN), peritoneal macrophages (PMØ) and alveolar macrophages (AMØ). *L. pneumophila* was opsonized in either (a) 50% pooled human serum (PHS) with excess immune IgG, (b) 25% PHS with sub-opsonic amounts of immune IgG, or (c) 25% PHS alone. After opsonization, bacteria were washed and mixed with indicated phagocytic cells. Uptake was recorded after 5, 15, 30 and 60 min. Symbols represent the means of three separate experiments; parentheses indicate the total ranges.

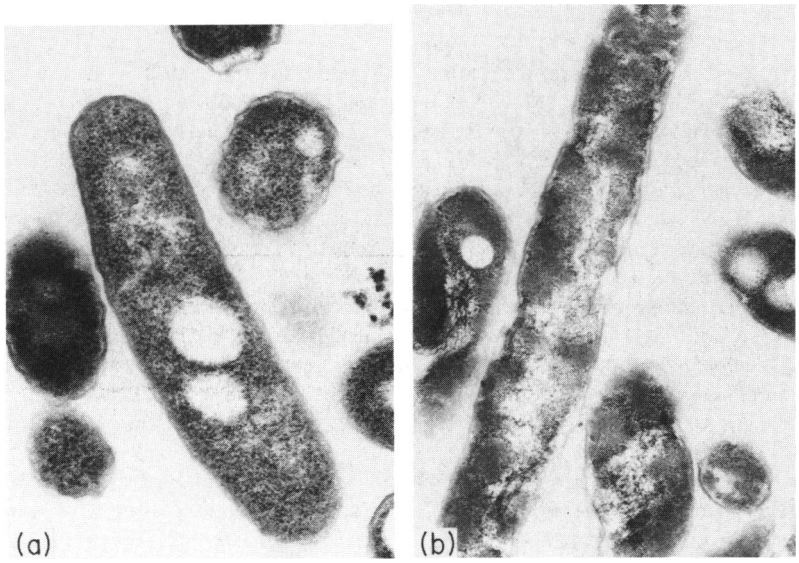

Figure 5. Ultrastructure of heated *L. pneumophila* compared with control bacteria kept on ice. *L. pneumophila* was kept at (a) 4° or (b) 100° for 1 hr and then prepared for electron microscopy. Note major cytoplasmic changes but intact cell wall structure of heated *L. pneumophila*. (Magnification × 26, 468.)

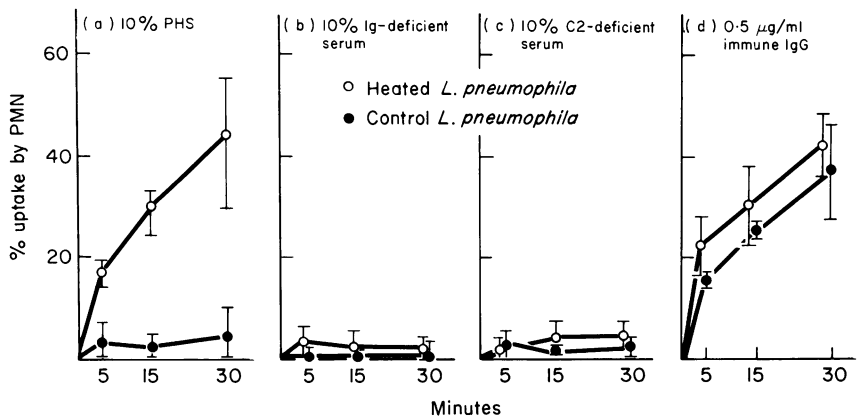

Figure 6. Effect of heat treatment on *L. pneumophila* interaction with complement and immune antibody. Heated (100° for 1 hr) and control (4° for 1 hr) *L. pneumophila* was opsonized in either (a) 10% pooled human serum (PHS), (b) 10% immunoglobulin (Ig)-deficient serum, (c) 10% serum without complement factor C2, or (d) in a solution of immune IgG in GHBSS. After opsonization, bacteria were washed and mixed with normal PMN. Uptake was recorded at 5, 15 and 30 min incubation. Symbols indicate the means of at least two separate experiments; bars represent ranges.

cally active C3b on the surface of heated *vs* control *Legionellae*, the amount of C3 fixed to the surface of the bacteria was quantitated by immunofluorescence. Surprisingly, heated and control *L. pneumophila* bound equal amounts of C3 when incubated in normal human serum. In three experiments, *L. pneumophila* showed a mean ± SE of 42 ± 4·0% C3 fixation and 44 ± 4·1% C3 fixation after pretreatments at 4° and 100°, respectively.

DISCUSSION

These studies show that both immunoglobulin and complement play important roles in the opsonization

of *L. pneumophila* (serogroup I). Either immune antibody or the complement system may provide the majority of opsonic molecules for this *Legionella* strain. Earlier reports also have pointed to the need for specific antibody; in the absence of such heat-stable opsonins, internalization of *L. pneumophila* by phagocytic cells proceeds at a much reduced rate (Kishimoto *et al.*, 1979; Horwitz & Silverstein, 1980b; Johnson *et al.*, 1979). The results of the present study suggest that many normal healthy donors, without a previous history of Legionnaires' disease, do not have levels of specific antibody sufficient to promote phagocytosis of *L. pneumophila*. Only six of 12 donors had detectable opsonic activity for *L. pneumophila* when their serum was tested at full strength. Further studies are needed to determine whether there is a correlation between the opsonic activity of human sera and antibody levels as measured by standard serological techniques.

The critical role of complement in mediating phagocytosis of *L. pneumophila* has received much less attention. It was previously noted that immune human serum, at a concentration of 10%, was opsonic for *L. pneumophila* only when the complement system remained intact (Horwitz & Silverstein, 1981). Additionally, it was observed that when fresh normal human serum was diluted to a concentration of 25%, C3 fixation to the surface of *L. pneumophila* occurred only when immune antibody was added to the serum (Horwitz & Silverstein, 1981). These observations have led these authors to conclude that both immune antibody and complement are needed for phagocytosis of *L. pneumophila*.

Results of the present study indicate that there are at least two distinct mechanisms for opsonization of *L. pneumophila*. Both require immune antibody, but only one is largely dependent upon the presence of complement. We found that *L. pneumophila* can be fully opsonized with immune antibody alone, albeit at relatively high concentration of IgG. On the other hand, the role of complement was clearly evident in experiments that demonstrated adequate *L. pneumophila* phagocytosis after incubation with subopsonic concentrations of immune antibody plus complement. It was noted that, in the presence of complement, only one-tenth the amount of immune antibody was required for *L. pneumophila* opsonization, and under these opsonic conditions phagocytosis was largely complement-mediated.

These observations may have relevance to clinical infection with *L. pneumophila*. During the initial phases of the development of humoral immunity, only limited amounts of immune antibody may be available, and clearance of the infecting organisms then would depend on the presence of an intact complement system. Such an important role for complement in the early stage of infection has recently been demonstrated in studies of *in vivo* clearance of *S. pneumoniae* (Brown *et al.*, 1982). Also, immunization with the serogroup I surface antigen has been shown to protect guinea-pigs against intraperitoneal challenge with *L. pneumophila*, indicating a potential protective effect of immune serum antibody (Elliott, Johnson & Helms, 1981). Additional studies are required, however, to define the importance of complement, as well as immune antibody, in the pathogenesis of Legionnaires' disease. Human tissue macrophages, especially those from alveolar spaces where Legionnaires' disease is initiated, were also used to measure *L. pneumophila* opsonization. Previous studies in our laboratories have revealed that, at least for some bacterial species, the need for opsonins may differ when human phagocytes other than peripheral blood PMN are used (Verbrugh *et al.*, 1982a, 1983). Results from this study, however, would indicate that human tissue macrophages require very similar levels and types of opsonins for the recognition of *L. pneumophila* as do PMN.

The interaction of *L. pneumophila* with the human complement system was of special interest. Many previous studies by several groups of investigators have shown that Gram-negative, as well as Gram-positive, bacteria are able to activate the complement system in various ways, and in so doing these organisms usually become opsonized. The routes of complement activation vary considerably and include the following:

(i) antibody-dependent interaction with the classical complement pathway (Verbrugh *et al.*, 1982b; Michael, Whitby & Landy, 1962);

(ii) antibody-independent activation of the alternative pathway (Dierich *et al.*, 1973; Gewurz, Shin & Mergenhagen, 1968; Verbrugh *et al.*, 1980);

(iii) a direct interaction with classical pathway C1 which is also independent of antibody (Clas & Loos, 1981; Leist-Welsh & Bjorson, 1982; Baker *et al.*, 1982);

(iv) an antibody-dependent activation of the alternative sequence of complement (Verbrugh *et al.*, 1982b; Edwards *et al.*, 1980).

Thus, even in the absence of immunoglobulins, many bacterial species, especially those with the characteristic outer membrane of Gram-negative organisms, are capable of activating human comple-

ment and, thereby, conferring opsonic recognition upon themselves. To our surprise, the *L. pneumophila* Philadelphia 1 strain was not capable of activating human complement unless immune antibody was also present. Studies with human sera genetically deficient in immunoglobulins or certain crucial complement factors further demonstrated that, even in the presence of immune antibody, the alternative complement pathway could not be activated by *L. pneumophila*. Furthermore, the interaction of this organism with complement remained strictly limited to antibody-dependent classical pathway activation and opsonization. Although the physicochemical ultrastructure of *L. pneumophila* has not been fully defined, several studies have indicated that lipopolysacharides, key activators of complement in the cell walls of other Gram-negative bacteria, are also present in the *L. pneumophila* envelope (Wong *et al.*, 1979). Interestingly, other biological tests for bacterial endotoxicity such as the rabbit pyrogenicity assay and the local Schwartzman reaction also showed *L. pneumophila* to be a comparatively weak reactor (Wong *et al.*, 1979). Encapsulation of *L. pneumophila* could also help explain its inertness relative to its interaction to the complement system. Encapsulated strains of other Gram-negative species, such as *E. coli*, are known to have a deminished ability to activate complement and bind C3 (van Dijk *et al.*, 1979). The ability to evade complement-mediated opsonization and, thereby, recognition by phagocytic cells may contribute to the high virulence of encapsulated *E. coli* (Howard & Glynn, 1971). Since *L. pneumophila* serogroup I has recently been shown to possess loosely bound capsular material (Hébert, Callaway & Ewing, 1984), a barrier to complement interaction may exist. In previous studies, heat-treatment has been used to remove capsular material from *E. coli* (van Dijk *et al.*, 1979) and we evaluated the effect of such treatment on *L. pneumophila*. Heating at $\geq 80°$ indeed increased the susceptibility of *Legionellae* to opsonization with diluted pooled normal serum. Interestingly, that treatment did not confer upon *L. pneumophila* the ability to activate the alternative complement pathway. In addition, the amounts of C3 that became fixed to *L. pneumophila* during opsonization did not change significantly. We therefore conclude that *L. pneumophila* possesses an unique surface structure that, in its native form, restricts complement activation as well as recognition by phagocytic cells. The lack of an antibody-independent mechanism of activating complement undoubtedly contributes to *L. pneumophila* high level of resistance to opsonization by normal, non-immune serum. In order to more fully define and understand the role of complement in host defence against the Legionnaires' disease bacterium, further studies are needed to quantitate and localize both the site and distribution of C3 molecules in relation to various structures of its outer membrane.

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REFERENCES

BAKER C.J., EDWARD M.S., WEBBE B.J. & KASPER D.L. (1982) Antibody-independent classical pathway-mediated opsonophagocytosis of type Ia, group B *Streptococcus*. *J. clin. Invest.* **69,** 394.

BROWN E.T., HOSEA S.W., HAMMER C.H., BRUCH C.G. & FRANK M.M. (1982) A quantitative analysis of the interactions of anti-pneumococcal antibody and complement in experimental pneumococcal bacteremia. *J. clin. Invest.* **69,** 85.

CLAS F. & LOOS M. (1981) Antibody-independent binding of the first component of complement (C1) and its subcomponent C1q to the S and R forms of *Salmonella minnesota*. *Infect. Immun.* **31,** 1138.

CLAWSON C.C. (1973) Platelet interaction with bacteria. III. Ultrastructure. *Am. J. Pathol.* **70,** 449.

DAISY J.A., BENSON C.E., MCKITRICK J. & FRIEDMAN H.M. (1981) Intracellular replication of *Legionella pneumophila*. *J. Infect. Dis.* **143,** 460.

DIERICH M.P., BITTER-SUERMANN D., KÖNIG W., HADDING U., GALANOS C. & RIETSCHEL E.T. (1973) Analysis of bypass activation of C3 by endotoxic LPS and loss of this potency. *Immunology*, **24,** 721.

VAN DIJK W.C., VERBRUGH H.A., VAN DER TOL M.E., PETERS R. & VERHOEF J. (1979) Role of *Escherichia coli* K capsular antigens during complement activation, C3 fixation, and opsonization. *Infect. Immun.* **25,** 603.

EDWARDS M.E., NICHOLSON-WELLER A., BAKER C.J. &

KASPER D.L. (1980) The role of specific antibody in alternative pathway-mediated opsonophagocytosis of type III, group B streptococcus. *J. exp. Med.* **151,** 1275.

ELLIOTT J.A., JOHNSON W. & HELMS C.M. (1981) Ultrastructural localization and protective activity of a high-molecular-weight antigen isolated from *Legionella pneumophila. Infect. Immun.* **32,** 822.

GEWURZ N., SHIN H.S. & MERGENHAGEN S.E. (1968) Interactions of the complement system with endotoxic lipopolysaccharide: consumption of each of six terminal complement components. *J. exp. Med.* **128,** 1049.

GIEBINK G.S., DEE T.H., KIM Y. & QUIE P.G. (1980) Alterations in serum opsonic activity and complement levels in pneumococcal disease. *Infect. Immun.* **29,** 1062.

HÉBERT G.A., CALLAWAY C.S. & EWING E.P. JR (1984) Comparison of *Legionella pneumophila, L. micdadei, L. bozimanii,* and *L. dumoffii* by transmission electron microscopy. *J. clin. Microb.* **19,** 116.

HOIDAL J.R., WHITE J.G. & REPINE J.E. (1979) Influence of cationic local anesthetics on the metabolism and ultrastructure of human alveolar macrophages. *J. Lab. clin. Med.* **93,** 857.

HORWITZ M.A. (1983) The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. *J. exp. Med.* **159,** 2108.

HORWITZ M.A. & SILVERSTEIN S.C. (1980a) Interaction of the Legionnaires' disease bacterium (*Legionella pneumophila*) with human phagocytes. II. Antibody promotes binding of *L. pneumophila* to monocytes but does not inhibit intracellular multiplication. *J. exp. Med.* **153,** 398.

HORWITZ M.A. & SILVERSTEIN S.C. (1980b) Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. *J. clin. Invest.* **66,** 441.

HORWITZ M.A. & SILVERSTEIN S.E. (1981) Interaction of the Legionnaires' disease bacterium (*Legionella pneumophila*) with human phagocytes. *J. exp. Med.* **153,** 386.

HOWARD C.J. & GLYNN A.A. (1971) The virulence for mice of strains of *E. coli* related to the effects of K antigens on their resistance to phagocytosis and killing by complement. *Immunology,* **20,** 767.

JOHNSON W., PESANTI E. & ELLIOTT J. (1979) Serospecific and opsonic activity of antisera to *Legionella pneumophila. Infect. Immun.* **26,** 698.

KIM Y., FRIEND P.S., DRESNER I.G., YUNIS E.J. & MICHAEL A.F. (1977) Inherited deficiency of the second component of complement (C2) with membrano proliferative glomerulonephritis. *Am. J. Med.* **62,** 765.

KISHIMOTO R.A., KASTELLO M.D., WHITE J.D., SHIREY F.G., MCGANN V.G., LARSON E.W. & HEDLUND K.W. (1979) *In vitro* interaction between normal cynomolgus monkey alveolar macrophages and Legionnaire's disease bacteria. *Infect. Immun.* **25,** 761.

LEIST-WELSH P. & BJORNSON A.B. (1982) Immunoglobulin-independent utilization of the classical complement pathway in opsonophagocytosis of *Escherichia coli* by human peripheral leucocytes. *J. Immunol.* **128,** 2643.

MICHAEL J.G., WHITBY J.L. & LANDY M. (1962) Studies on natural antibodies to Gram-negative bacteria. *J. exp. Med.* **115,** 131.

PETERSON P.K., VERHOEF J., SCHMELING D. & QUIE P.G. (1977) Kinetics of phagocytosis and bacterial killing by human polymorphonuclear leucocytes and monocytes. *J. Infect. Dis.* **136,** 502.

PETERSON P.K., WILKINSON B.J., KIM Y., SCHMELING D. & QUIE P.G. (1978) Influence of encapsulation on staphylococcal opsonization and phagocytosis by human polymorphonuclear leucocytes. *Infect. Immun.* **19,** 943.

ROORD J.J., DAHA M., KUIS W., VERBRUGH H.A., VERHOEF J., ZEGERS B.J.M. & STOOP J.W. (1982) Inherited deficiency of the third component of complement associated with recurrent pyogenic infections, circulating immune complexes, and vasculitis in a Dutch family. *Pediatrics,* **72,** 81.

STOSSEL T.P., FIELD R.J., GITLIN J.D., ALPER C.A. & ROSEN F.S. (1975) The opsonic fragment of the third component of human complement (C3). *J. exp. Med.* **141,** 1329.

VERBRUGH H.A., VAN DIJK W.C., PETERS R., VAN ERNE M.E., DAHA M.R., PETERSON P.K. & VERHOEF J. (1980) Opsonic recognition of staphylococci mediated by cell wall peptidoglycan: antibody-independent activation of human complement and opsonic activity of peptidoglycan antibodies. *J. Immunol.* **124,** 1167.

VERBRUGH H.A., VAN DIJK W.C., VAN DER TOL M.E., PETERS R., PETERSON P.K. & VERHOEF J. (1979) Quantitation of the third component of human complement attached to the surface of opsonized bacteria: Opsonin-deficient sera and phagocytosis-resistant strains. *Infec. Immun.* **26,** 808.

VERBRUGH H.A., HOIDAL J.R., NGUYGEN B-Y.T., VERHOEF J., QUIE P.G. & PETERSON P.K. (1982a) Human alveolar macrophages cytophilic immunoglobulin G-mediated phagocytosis of protein A-positive staphylococci. 1982. *J. clin. Invest.* **69,** 63.

VERBRUGH H.A., KEANE W.F., HOIDAL J.R., FREIBERG M.R., ELLIOTT G.R. & PETERSON P.K. (1983) Peritoneal macrophages and opsonins: antibacterial defense in patients undergoing chronic peritoneal dialysis. *J. Infect. Dis.* **147,** 1018.

VERBRUGH H.A., PETERSON P.K., NGUYGEN B-Y.T., SISSON S.P. & KIM Y. (1982b) Opsonization of encapsulated *Staphylococcus aureus*: the role of specific antibody and complement. *J. Immunol.* **129,** 1681.

WONG K.H., EWING E.P. JR, CALLAWAY C.S. & PEACOCK W.L. JR (1980) Intracellular multiplication of Legionella pneumophila in cultured human embryonic lung fibroblasts. *Infect. Immun.* **28,** 1014.

WONG K.H., MOSS C.W., HOCHSTEIN D.H., ARKO R.J. & SCHALLA W.O. (1979) 'Endotoxicity' of the Legionnaires' disease bacterium. *Ann. Inter. Med.* **90,** 624.