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Adherence of *Legionella pneumophila* to Guinea Pig Peritoneal Macrophages, J774 Mouse Macrophages, and Undifferentiated U937 Human Monocytes: Role of Fc and Complement Receptors

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***Legionella pneumophila*, the causative agent of Legionnaires' disease, is a facultative intracellular pathogen of alveolar macrophages. Although previous studies have demonstrated that specific antibody facilitates uptake of *L. pneumophila* by phagocytic cells, the role of complement has been unclear. Thus, we have examined the relative contributions of Fc gamma- and complement receptor-mediated adherence to guinea pig peritoneal macrophages, U937 human monocytic cells, and J774 mouse macrophage cells. Opsonization of *L. pneumophila* (Philadelphia 2) with polyclonal immunoglobulin G promoted maximum adherence to guinea pig macrophages. In contrast, incubation in the presence of 20% fresh nonimmune human serum from a single donor did not promote adherence. The results obtained with U937 and J774 cells paralleled those obtained with guinea pig macrophages. In the absence of specific antibody, opsonization with guinea pig complement did not enhance adherence of the Philadelphia 1, Philadelphia 2, or Knoxville strain. However, when complement was added to heat-inactivated, specific antiserum, a fourfold increase in the number of adherent organisms was observed. Blocking studies utilizing membrane receptor-specific monoclonal antibodies demonstrated that both Fc and complement receptors mediated adherence of organisms treated with complement in the presence of specific antibody. These results suggest that complement augments adherence of *L. pneumophila* only when acting in concert with specific antibody.**

Legionella pneumophila is a fastidious, gram-negative, aerobic bacillus commonly isolated from lakes, streams, potable water supplies, and cooling towers (3, 7, 26). Transmission of Legionnaires' pneumonia is believed to involve inhalation of organisms aerosolized from contaminated potable water supplies. Since studies have indicated that, in its aquatic environment, *L. pneumophila* is a parasite of freshwater amoebae and protozoa (9, 37), it has been suggested that bacterium-filled vesicles released from infected amoebae are the infective particles for humans (37). In the human lung, *L. pneumophila* is an intracellular pathogen which replicates within alveolar macrophages (44). Although phagocytosis by alveolar macrophages is a critical step in establishment of infection by this organism, there is still some question regarding the role of serum opsonins in mediating phagocytosis. Several investigators have shown that specific antibody facilitates uptake of *L. pneumophila* by phagocytic cells (15, 16, 19, 28, 42, 46); however, the potential contribution of complement is not as certain. In an earlier study from this laboratory, investigators found that serogroup-specific antiserum, but not complement, augmented phagocytosis of *L. pneumophila* by rat alveolar macrophages and mouse peritoneal macrophages (16). However, other investigators have found that bound C3 fragments interact with complement receptors on human peripheral blood monocytes (29) and HL-60 human monocytic cells (23), mediating uptake of *L. pneumophila* in the absence of specific antibody.

To confirm and extend the results of these previous studies, we have investigated the relative contributions of Fc- and complement receptor-mediated adherence to guinea pig peritoneal macrophages, the U937 human monocytic cell line, and the J774 mouse macrophage cell line. Guinea pigs are readily susceptible to infection by virulent strains of *L. pneumophila*, and macrophages from these animals are thought to be an important model for studying probable interactions of this organism with human alveolar macrophages. The U937 cell line has been shown by other investigators to support the growth of virulent strains of *L. pneumophila* (17, 30). U937 cells are similar to human alveolar macrophages in that they express very few type 1 complement receptors (CR1), and CR3 expression on these cells (compared with that on blood monocytes) is relatively weak (11, 38). In the present study, cell surface receptor monoclonal antibodies (MAbs) were used confirm receptor binding specificity of opsonized *L. pneumophila* to the U937 cell line. The J774 cell line was isolated from a BALB/c/NIH mouse which had acquired a reticulum cell sarcoma (33). These cells exhibit the properties of peritoneal macrophages, and preliminary experiments using specific MAbs confirmed that these cells express CR1 and a relatively high level of CR3.

The results of this study suggest that (i) in the absence of specific antibody, complement does not promote adherence of *L. pneumophila* to mononuclear phagocytes; (ii) opsonizing antibody significantly enhances adherence to guinea pig peritoneal macrophages, the U937 human monocytic cell line, and the J774 mouse macrophage cell line; and (iii) complement and specific antibody act in concert to augment adherence of *L. pneumophila* to U937 cells.

MATERIALS AND METHODS

Organisms. The *L. pneumophila* serogroup 1 strains Philadelphia 2, Philadelphia 1, and Knoxville were obtained

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from the Centers for Disease Control, Atlanta, Ga. A virulent culture of the Philadelphia 2 strain was derived as previously described and was maintained on charcoal yeast extract agar (6). Unencapsulated *Staphylococcus aureus* EV was obtained from Frank Kapral, Ohio State University, Columbus. For some adherence assays, organisms were labeled with fluorescein isothiocyanate (FITC) by the method of Korhonen et al. (21), with two modifications. The wash buffer used to remove unbound FITC did not contain Tween 20, and the organisms were used immediately after conjugation. Control experiments showed that FITC conjugation did not inhibit antibody binding by the organisms, nor did it inhibit complement deposition either in the presence or absence of specific antibody.

Mononuclear cells. Unstimulated peritoneal macrophages were harvested from outbred guinea pigs by peritoneal lavage, distributed into wells of a 48-well tissue culture dish, and cultured as monolayers in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered RPMI medium supplemented with heat-inactivated fetal calf serum (FCS) (20%, vol/vol), penicillin (100 U/ml), and streptomycin (100 μg/ml). The U937 monocytic cell line was a gift from Morris Dailey, Department of Pathology, University of Iowa, Iowa City, and was maintained in replicative, nonadherent culture. The J774 macrophage cell line was a gift from Mary Wilson, Department of Internal Medicine, University of Iowa, and was maintained in monolayer culture. Both cell lines were cultured in HEPES-buffered RPMI medium supplemented with heat-inactivated FCS (10%, vol/vol), glutamine (4 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). All cells and cell lines were incubated in a humid atmosphere of 5% CO_2. The origins of the U937 (32, 40) and J774 (33) cell lines have been previously described.

***L. pneumophila*-specific antibodies.** Preparation of rabbit antiserum to *L. pneumophila* serogroup 1 (Philadelphia 2) has been previously described (16). The immunoglobulin G (IgG) fraction was purified by affinity chromatography on a HiPAC Protein G LTQ column (Chromatochem, Missoula, Mont.) according to the manufacturer's instructions. The elution buffer was 0.1 M glycine in 2% (vol/vol) acetic acid, pH 3.7. $F(ab)'_2$ fragments of the purified rabbit IgG were prepared by overnight digestion with immobilized pepsin (Pierce Chemical Co., Rockford, Ill.) at pH 4.2. Contaminating, uncleaved IgG was removed from the $F(ab)'_2$ preparation by a second application to the protein G column and by collection of material that did not bind to the column. Purity of the whole-antibody and $F(ab)'_2$ preparations was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Preparation of a mouse MAb specific for the F-1 antigen from *L. pneumophila* serogroup 1 (Philadelphia 2) has been previously reported (20). This antibody, designated MAb 1E6, is of the IgG1 isotype. The 1E6-secreting hybridoma was grown in HB101 medium by the University of Iowa Tissue Culture/Hybridoma Facility, and the resulting supernatant was dialyzed extensively against distilled water and lyophilized before use.

Anti-*L. pneumophila* antibodies were used at the following concentrations: immune rabbit serum (microagglutination titer of 1/256), 1/450 dilution; rabbit polyclonal IgG, 5 μg/ml; rabbit polyclonal IgG $F(ab)'_2$ fragments, 10 μg/ml; and MAb 1E6, 0.2 μg/ml.

Blocking antibodies. MAbs 10.1 (IgG1) and IV.3 (IgG2b) are directed against the high- (group I) and low (group II)-affinity Fc gamma receptors (8, 22), respectively. MAb OKM1 (IgG2b) is directed against CR3 (45). MAb 10.1 ascites fluid was generously provided by Nancy Hogg, Imperial Cancer Research Fund. MAb IV.3 IgG and Fab fragment were purchased from Medarex, Inc. (West Lebanon, N.H.). MAb OKM1 IgG was purchased from Ortho Diagnostics, Inc. (Raritan, N.J.).

Sera. Guinea pig complement serum was obtained from Sigma Chemical Co. (St. Louis, Mo.) and stored at −70°C until use. FCS was obtained from the University of Iowa Tissue Culture/Hybridoma Facility and stored at −20°C until use. Human serum was obtained from a nonimmune, healthy volunteer and used fresh. When necessary, serum was heat inactivated by being heated for a minimum of 1 h at 56°C to remove complement activity.

Staphylococcal delta toxin. *Staphylococcus aureus* EV was grown in a yeast extract-Trypticase broth (1% [wt/vol] yeast extract, 1.7% [wt/vol] Trypticase, 0.5% [wt/vol] sodium chloride, 0.25% [wt/vol] K_2HPO_4) for 18 to 24 h at 37°C on a gyratory shaker. A crude delta toxin preparation was obtained from the culture supernatant by an abbreviated version of the method of Yoshida (47). Briefly, the supernatant was harvested, acidified to pH 4 with acetic acid, and precipitated with ammonium sulfate. The precipitate was dissolved in distilled water, and the resulting solution was adjusted to pH 9 with ammonium hydroxide, dialyzed against distilled water, and lyophilized. The crude toxin was dissolved in potassium phosphate-buffered saline (KPBS), pH 7.6, before use. Preliminary experiments showed that the crude delta toxin preparation did not decrease *L. pneumophila* viability after a 24-h exposure.

Adherence to guinea pig macrophages. Twenty-four-hour cultures of *L. pneumophila* were suspended to a density of 5×10^7 to 1×10^8 CFU/ml in antibiotic-free tissue culture medium containing 20% (vol/vol) FCS or fresh nonimmune human serum. Control experiments showed that the use of xenogeneic serum in the tissue culture medium did not significantly reduce viability of the guinea pig macrophages. (Loss of viability over 2 h was less than 10%.) In some experiments, the organisms were opsonized with whole antibody or $F(ab)'_2$ fragments for 30 min before addition to the monolayers. Macrophages (ca. 5×10^3 per well), previously washed and incubated for a minimum of 2 h in antibiotic-free medium, were coincubated with bacteria at a ratio of ca. 10,000 bacteria per macrophage for 2 h at 37°C and washed five times with cold medium. This washing procedure was found to be sufficient to minimize adherence of organisms to plastic surfaces. Macrophages were lysed by the addition of 0.5 ml of *S. aureus* delta toxin solution (final concentration, ca. 5 mg/ml). A 150-μl volume of the macrophage lysate was removed, and bacteria were enumerated by the Gen-Probe assay (Gen-Probe Inc., San Diego, Calif.) according to the manufacturer's instructions. The number of cell-associated *L. pneumophila* per milliliter of macrophage lysate was determined by comparison to a standard curve derived from plating on charcoal yeast extract agar.

Adherence to U937 cells. U937 cells were suspended in medium containing 20% heat-inactivated FCS and distributed into wells of a 48-well tissue culture plate. Anti-receptor or isotypic control MAbs were added at 30 μg/ml (total volume, 0.5 ml), and the plates were incubated for 45 to 60 min on ice. To ensure maximal binding to cell surface receptors, unbound antibody was not washed from the cells. FITC-conjugated bacteria were preincubated with serum opsonins at 37°C and added directly to the U937 cells at a bacterium-to-cell ratio of ca. 10. Monocytic cells were coincubated with bacteria for 45 to 60 min at 37°C. The U937 cells were diluted in 50 ml of fixative to prevent nonspecific

bacterial adherence, stained with propidium iodide, and separated from nonadherent bacteria on a Coulter EPICS 753 flow cytometer (Coulter Electronics, Inc., Hialeah, Fla.). The adherence index (number of adherent bacteria per 100 cells) was determined by examining a minimum of 100 cells on an Olympus BH2 fluorescent microscope (Olympus Optical Co., Ltd., Tokyo, Japan). All adherence indices represent the mean of triplicate samples, except for the *S. aureus* controls, for which the adherence indices were determined from single wells.

Adherence to J774 cells. J774 cells were distributed into wells of a 48-well tissue culture plate and cultured as monolayers. Before use in adherence assays, the cells were washed and the culture medium was replaced with antibiotic-free medium containing 1 μg of cytochalasin D (Sigma) per ml and 20% heat-inactivated FCS. The cells were incubated in the presence of cytochalasin D for a minimum of 1 h before use. Bacteria were preincubated with serum opsonins at 37°C for 45 min and added directly to the J774 cells at a bacterium-to-cell ratio of ca. 250. The macrophages were coincubated with bacteria for 60 min at 37°C and then washed three times with KPBS. Adherent *Legionella* organisms were detected by an indirect fluorescent-antibody assay using MAb 1E6 as the primary antibody. Briefly, a 0.5-ml portion of 1E6 antibody solution (final concentration, 100 μg/ml) was added to each well and allowed to stand for 40 min. The macrophages were then washed twice with KPBS, 0.5 ml of FITC-conjugated anti-mouse IgG (Cappell-Organon Teknika Corp., West Chester, Pa.) (final concentration, 43.5 μg/ml) was added, and the mixture was allowed to stand for 45 min. The macrophages were washed twice with KPBS and stained with propidium iodide. The monolayers were kept on ice throughout the fluorescent-antibody assay to prevent internalization of adherent organisms. The *S. aureus* control organisms were directly conjugated with FITC before use in the assay. The adherence indices were determined in the same manner as for the U937 cells and represent the mean of triplicate samples.

RESULTS

Adherence to guinea pig macrophages. A previous study from our laboratory suggested that serogroup-specific antiserum is required for optimum phagocytosis of *L. pneumophila* (Philadelphia 2) by rat alveolar macrophages and mouse peritoneal macrophages (16). These studies were extended to examine the specific contribution of complement and antibody toward adherence to guinea pig peritoneal macrophages. As shown in Fig. 1, maximum adherence was observed following opsonization with whole IgG. When $F(ab)'_2$ fragments were used for opsonization, the degree of adherence was reduced to approximately the same level observed for control organisms incubated in FCS. These results implicated Fc gamma receptor-mediated phagocytosis as an important mechanism of uptake of this organism. The number of adherent organisms in the presence of a source of complement (20% human serum), however, was minimal and not significantly greater than that observed for control organisms incubated in FCS (Fig. 1), which was found not to contain active complement activity. (In addition, similar results were obtained when heat-inactivated FCS was used [data not shown].) These results suggest that phagocytosis of Philadelphia 2 organisms by guinea pig macrophages is not mediated by cell surface complement receptors.

Adherence to U937 cells. Our next experiments were carried out to examine the effect of serum opsonins on adherence of *L. pneumophila* to the U937 human monocytic cell line. U937 cells have been shown to support the growth of virulent strains of *L. pneumophila* (17, 30) and distinguish between virulent and avirulent cells (30). However, the capacity of antibody and complement to mediate adherence of *L. pneumophila* to these cells has not been studied. As shown in Table 1, an adherence index of 255 was obtained following opsonization with anti-*L. pneumophila* MAb (MAb 1E6). In contrast, an adherence index of 2 was obtained for organisms incubated in the presence of normal complement serum (5 50% complement units [CH_{50}]/ml). This value was not significantly different from that observed for control organisms incubated in the presence of heat-inactivated serum. Thus, the results obtained with the U937 cells paralleled those obtained with guinea pig peritoneal macrophages (Fig. 1), indicating that complement does not enhance adherence of *L. pneumophila* to phagocytic cells in the absence of specific antibody. This finding is not strain specific because similar results were obtained for two other serogroup 1 isolates, Knoxville (Table 1) and Philadelphia 1 (data not shown). The inability of complement to enhance adherence of *L. pneumophila* to U937 cells does not appear to be due to the presence of inactive complement receptors on the U937 cells since, in a control experiment, opsoniza-

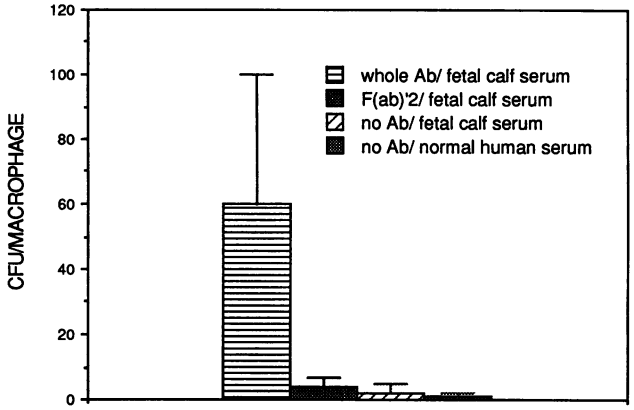

FIG. 1. Effect of serum opsonins on adherence of *L. pneumophila* to guinea pig peritoneal macrophages. Values are expressed as the means ± standard deviations of two to five experiments. Ab, antibody.

TABLE 1. Effect of serum opsonins on adherence of *L. pneumophila* to the U937 monocytic cell line

Strain or organism	Opsonization[a]	Adherence index (mean ± SD)
Philadelphia 2	MAb 1E6	255 ± 62
	N serum[b]	2 ± 3
	N serum (HI)	3 ± 1
Knoxville	N serum[c]	2 ± 0
	N serum (HI)	3 ± 1
S. aureus	N serum[d]	>344
	N serum (HI)	48

[a] Abbreviations: N, normal; HI, heat inactivated.
[b] Complement serum added at 5 CH50 U/ml.
[c] Complement serum added at 15 CH50 U/ml.
[d] Complement serum added at 10 CH50 U/ml.

TABLE 2. Fc receptor binding specificity of MAb 1E6-opsonized *L. pneumophila* in a U937 cell adherence assay

Blocking antibody (specificity)	Adherence index (mean ± SD)	% Inhibition of adherence
Isotypic control	300 ± 106	
10.1 (FcγRI)	292 ± 101	3
IV.3 (FcγRII)	8 ± 10	97
10.1 + IV.3	6 ± 7	98

tion of unencapsulated *S. aureus* EV with guinea pig complement serum (10 CH_{50}/ml) enhanced adherence approximately sevenfold.

Previous studies have shown that mouse IgG1 immune complexes bind preferentially to the low-affinity Fc gamma receptors on human leukocytes (1, 22). We wanted to confirm that immune complexes formed by opsonization of *L. pneumophila* with MAb 1E6 (an IgG1 antibody) also bind to the low-affinity (group II) Fc gamma receptor on U937 cells. The results are shown in Table 2. Anti-FcγRI MAb 10.1 did not significantly block adherence, whereas anti-FcγRII MAb IV.3 blocked adherence by a mean of 97%. Thus, IgG1-opsonized *L. pneumophila* interact with Fc gamma receptors on U937 cells in a manner similar to that described in previous reports of binding of immune complexes to U937 cells and peripheral blood monocytes (22).

Since complement, in the absence of specific antibody, did not enhance adherence of *L. pneumophila* to guinea pig peritoneal macrophages or U937 cells, we wanted to determine whether complement promoted adherence when added in the presence of specific antibody. Addition of 15 CH_{50}/ml of complement to heat-inactivated immune rabbit serum enhanced adherence to the U937 cells approximately fourfold, and a 50% reduction in adherence of these organisms was observed following incubation of U937 cells with OKM1 anti-CR3 MAb (Table 3). The degree of blocking observed when anti-FcγRII and anti-CR3 MAbs were used in combination (82%) was more pronounced than that observed when either antibody was used alone. When both antibodies were used together, the U937 cells were incubated with MAb IV.3 IgG prior to their incubation with OKM1 IgG. Since both MAbs are of the same subtype (IgG2b), incubation with MAb IV.3 prior to incubation with MAb OKM1 minimizes the possibility that the added degree of blocking observed with OKM1 is simply steric hindrance of Fc receptor-mediated interactions. Thus, both Fc gamma receptors and complement receptors mediate adherence of antibody- and complement-coated *L. pneumophila* to U937 cells.

Adherence to J774 cells. Other investigators (23, 29) have reported that phagocytosis of *L. pneumophila* (Philadelphia 1) by human monocyte-derived macrophages and differentiated HL-60 cells is mediated by both CR1 and CR3. Since, in the present study, most experiments to this point utilized undifferentiated U937 cells, which do not express CR1, the next experiments were carried out to examine the possibility of complement receptor-mediated adherence to cells which do express CR1. For these experiments, the J774 mouse macrophage cell line was utilized. CR1 expression by the J774 cells was confirmed with flow cytometry by using the specific MAb 8C12 (14, 18) (data not shown). In addition, flow cytometry indicated that these cells express a relatively high level of CR3 (detected by MAb M1/70 [2]) (data not shown). Use of phagocytic cells expressing a high density of complement receptors is useful to examine the possibility of recognition of low-density complement ligands. The capacity of CR3 on J774 cells to mediate phagocytosis was recently demonstrated by Hall et al. (12) in a study concerning incorporation of cell surface receptors into the phagocytic vacuole following phagocytosis of the intracellular parasite *Trypanosoma cruzi*. As shown in Table 4, an adherence index of 78 was obtained for Philadelphia 2 organisms opsonized with heat-inactivated polyclonal antiserum. In contrast, an adherence index of 2 was obtained for organisms incubated in the presence of 40% normal complement serum. This value was not significantly different from that observed for control organisms incubated with heat-inactivated serum. Similar results were obtained with the Knoxville strain. In control experiments, addition of 40% normal complement serum to *S. aureus* enhanced adherence to the J774 cells approximately threefold.

TABLE 3. Effect of complement on adherence of *L. pneumophila* to U937 cells in the presence of specific antibody

Opsonization[a]	Blocking antibody (specificity)	Adherence index	% Inhibition of adherence
HI immune serum	None	34 ± 3	
HI immune serum + complement[b]	None	157 ± 53	
	OKM1 (CR3)	78 ± 26	50
	IV.3 (FcγRII)	75 ± 25	52
	IV.3 + OKM1	28 ± 12	82

[a] Abbreviations are defined in Table 1, footnote *a*.
[b] Complement serum added at 15 CH50 U/ml.

TABLE 4. Effect of serum opsonins on adherence of *L. pneumophila* to the J774 mouse macrophage cell line

Strain or organism	Opsonization[a]	Adherence index (mean ± SD)
Philadelphia 2	HI immune serum	78 ± 4
	N serum[b]	2 ± 2
	N serum (HI)	0 ± 0
Knoxville	N serum[b]	5 ± 3
	N serum (HI)	3 ± 3
S. aureus	N serum[c]	66 ± 13
	N serum (HI)	18 ± 14

[a] Abbreviations are defined in Table 1, footnote *a*.
[b] Serum used at 40%.
[c] Serum used at 20%.

DISCUSSION

In this study, guinea pig peritoneal macrophages, the U937 human monocytic cell line, and the J774 mouse macrophage cell line were used to investigate the role of Fc gamma and complement receptors in mediating adherence of *L. pneumophila*. The guinea pig is the recognized animal model for experimental legionellosis. Guinea pigs are very susceptible to infection by virulent strains of *L. pneumophila*, and when the organism is given by an aerosol or intratracheal route, these animals develop lung lesions which histopathologically resemble those seen in humans. Thus, macrophages from these animals are thought to be a useful model for studying probable interactions of *L. pneumophila* with human alveolar macrophages. The U937 cell line was established from a patient with a histiocytic lymphoma (40). These cells exhibit

characteristics of blood monocytes (32, 40) and have been shown by other investigators to support the growth of virulent strains of *L. pneumophila* (17, 30). This cell line was chosen for use in this study because of its similarities to human alveolar macrophages in complement receptor expression. Human alveolar macrophages express very little, if any, CR1 (24, 27), and CR3 expression on these cells, compared with that on blood monocytes, is relatively weak (10, 24, 27). Correspondingly, unstimulated U937 cells express negligible amounts of CR1 and low levels of CR3 (11, 38). The functional activity of CR3 on uninduced U937 cells was demonstrated in a study by Reisinger et al. (34) on the role of complement in mediating adherence of human immunodeficiency virus type 1. The J774 mouse macrophage cell line was derived from a BALB/c/NIH mouse which had acquired a reticulum cell sarcoma (33). These cells are known to exhibit the adherent, cytologic, and phagocytic properties of mouse peritoneal macrophages, and preliminary experiments confirmed that these cells express CR1 and a relatively high level of CR3. The utility of this cell line for studying complement receptor-mediated adherence was recently demonstrated during a study of receptor sorting during phagocytosis of the intracellular protozoan parasite *Trypanosoma cruzi* (12).

Our data suggest that, in the absence of specific antibody, complement does not enhance adherence of *L. pneumophila* to mononuclear phagocytes. Incubation of *L. pneumophila* in the presence of 20% normal human serum did not augment adherence to guinea pig peritoneal macrophages (Fig. 1). In addition, treatment of organisms with normal guinea pig complement serum did not enhance adherence to the U937 human monocytic cell line (Table 1) or the J774 mouse macrophage cell line (Table 4). These results contradict those of Payne and Horwitz (29), who found that, in the presence of nonimmune human serum, monocyte CR1 and CR3 receptors mediate uptake of *L. pneumophila* (Philadelphia 1) following complement C3 deposition via the alternative complement cascade. Other investigators (25, 42), however, have shown that complement activation by Philadelphia 1 organisms occurs primarily via the classical pathway and that the opsonic capacity of individual, nonimmune serum varies considerably. Verbrugh et al. (42), utilizing human serum deficient in immunoglobulin or specific complement components, found that opsonization of Philadelphia 1 organisms by nonimmune serum is dependent on antibody-mediated activation of the classical pathway. Correspondingly, while pooled normal human serum weakly promotes phagocytosis by polymorphonuclear leukocytes, agammaglobulinemic serum does not. Mintz et al. have also demonstrated that complement activation by purified lipopolysaccharide (25) and major outer membrane (24a) fractions from Philadelphia 1 organisms occurs primarily by the classical (antibody-dependent) pathway in nonimmune human serum. Furthermore, the antibody mediating this classical activation was found to be naturally occurring IgM.

It is possible that the disparity between our results and those of other investigators (23, 29) is due to complement activation by IgM antibody in some sources of nonimmune serum. We have utilized high-complement-titer guinea pig serum for most of our assays. Guinea pig serum exhibits significantly greater hemolytic activity than does human serum; thus, we were able to dilute the guinea pig serum extensively, minimizing the presence of naturally occurring antibody. Since IgM antibody is not present in normal bronchial fluid (35), addition of dilute serum as a source of complement may more closely approximate the potential opsonic molecules available to the organism at the very early stages of infection than would addition of high concentrations of pooled human serum. In one experiment (Fig. 1), we tested 20% nonimmune human serum from a single donor and found it to be nonopsonic. Verbrugh et al. (42) found that only one half of the normal donor sera that they tested exhibited detectable opsonic activity to *L. pneumophila* (Philadelphia 1) when used full strength.

Second, we have shown that opsonizing antibody significantly enhances adherence to guinea pig peritoneal macrophages (Fig. 1), U937 human monocytic cells (Table 2), and J774 mouse macrophage cells (Table 4). Results of blocking with MAbs specific for the high (group I)- and low (group II)-affinity Fc receptors demonstrate that immune complexes formed by opsonization of *L. pneumophila* with a mouse IgG1 MAb bind specifically to the low-affinity Fc receptors on human cells (Table 2). This result correlates with that of other investigators who have studied the specificity with which murine IgG subclasses interact with Fc gamma receptors on human leukocytes (1, 22). The capacity of opsonizing antibody to augment phagocytosis of *L. pneumophila* by mononuclear phagocytes has been previously reported (15, 16, 19, 28, 46).

Finally, our results demonstrate that while specific antibody alone is sufficient to mediate adherence, complement acts to enhance adherence when added in the presence of specific antibody. Addition of fresh guinea pig serum to heat-inactivated immune rabbit serum significantly increased adherence to U937 cells. Correspondingly, treatment with OKM1 (anti-CR3) MAb resulted in a 50% reduction in adherence of antibody- and complement-coated organisms (Table 3). Normal human serum has also been shown to enhance phagocytosis of antibody-coated *L. pneumophila* by polymorphonuclear leukocytes (42). Thus, C3 fragments deposited during classical activation by specific antibody appear to interact with complement receptors on the surface of phagocytic cells.

Although some studies on the Philadelphia 1 strain of *L. pneumophila* have indicated that complement activation in normal serum is dependent on antibody-mediated activation of the classical pathway (25, 42), previous investigations have also indicated that the extent of complement binding in normal serum is dependent on the particular strain or isolate examined. Plouffe et al. (31) examined two serogroup 1 subtypes and observed marked differences in their survival in normal human serum, which correlated with surface binding of complement components. Summersgill et al. (39) have also found variations in the ability of serogroup 1 isolates to bind complement component C3. In our assays, we could not detect, by immunofluorescence, bound C3 fragments on the surface of Philadelphia 2 organisms following incubation in guinea pig complement serum. However, complement C3 was consistently detected on the surface of a Knoxville isolate. Treatment of this Knoxville isolate with guinea pig complement serum, however, did not enhance adherence to U937 cells (Table 1), suggesting that C3 fragments deposited during incubation in normal serum, in contrast to those deposited during incubation in immune serum, are unavailable for interaction with receptors on phagocytic cells. There are two possible explanations for this observation. Either the quantity of C3 fragments deposited in the absence of specific antibody is below that necessary for recognition by cell surface complement receptors or bound C3 fragments deposited in the absence of specific antibody are deposited at sites distinct from those where they are deposited during activation by specific antibody and

are masked by cell surface components. A previous investigation supports the latter possibility (42). Heating *L. pneumophila* (Philadelphia 1) to temperatures equal to or greater than 80°C prior to treatment with 10% pooled nonimmune serum significantly increases uptake by polymorphonuclear leukocytes. This heat treatment does not increase uptake of organisms opsonized with immune antibody, nor does it increase the amount of C3 deposited on the bacteria, leading investigators to conclude that *L. pneumophila* possesses a unique surface structure, possibly capsular material, that restricts recognition by phagocytic cells. Capsules on other bacterial species have been shown to block interaction of covalently bound C3 fragments with complement receptors (4, 43); however, the presence of extracellular polysaccharide material on *L. pneumophila* is uncertain. Johnson et al. (16) have isolated a loosely bound, high-molecular-weight surface component (F-1 fraction) and have suggested that it functions as a capsule or slime layer. Other investigators (6, 13, 41) have identified a ruthenium red-positive polysaccharide capsule on *L. pneumophila*, but other studies have failed to confirm this finding (36). Thus, experiments are under way in our laboratory to characterize the sites of C3 deposition on *L. pneumophila* strains in both the presence and absence of specific antibody, specifically in relation to the F-1 antigen and other cell surface structures.

In conclusion, we propose that during the very early stages of infection, before a specific humoral response is mounted, phagocytosis of *L. pneumophila* by alveolar macrophages may occur by a nonopsonic mechanism. Later in the course of infection, specific antibody, acting alone or in combination with complement components, should significantly enhance phagocytosis and is the principal mediator of uptake of *L. pneumophila* by macrophages.

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