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Antibody-Independent Binding of Complement Component C1q by *Legionella pneumophila*

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Incubation of *Legionella pneumophila* Philadelphia 1 in normal human serum depleted of either classical-pathway component C1q or alternative-pathway component factor B resulted in activation of the complement system. Experiments focused on the role of the classical pathway in complement activation revealed that legionellae bound C1q independently of antibody. Purified preparations of *L. pneumophila* major outer membrane protein but not serogroup 1 lipopolysaccharide bound C1q independently of antibody. This suggests that antibody-independent binding of C1q by *L. pneumophila* can result in activation of the classical pathway in normal human serum and that major outer membrane protein may be a C1q acceptor on the *L. pneumophila* cell surface.

Legionella pneumophila is a gram-negative, facultative intracellular bacterium that can enter and multiply within a variety of eukaryotic cells including mononuclear phagocytes (15, 26), cultured human and animal cells (5, 27), and free-living amoebae (7, 14, 25). Although the mechanism(s) by which *L. pneumophila* enters cultured mammalian cell lines and amoebae is not completely understood, it is well established that the opsonin C3 (as activated C3b) and phagocyte complement receptors CR1 and CR3 participate in the uptake of *L. pneumophila* by human monocytes and alveolar macrophages (4, 26).

Several years ago, Bellinger-Kawahara and Horwitz (4) demonstrated that the *L. pneumophila* major outer membrane protein (MOMP) can activate complement and selectively bind C3 during incubation of whole cells of *L. pneumophila* Philadelphia 1 in normal human serum (NHS). Furthermore, they showed that MOMP-containing liposomes incubated in NHS could bind C3 and attach to human monocyte monolayers in a complement-dependent manner. These results indicated that MOMP acted as a complement activator and that MOMP played a critical role in the complement-dependent uptake of legionellae by human mononuclear phagocytes. Of interest, Bellinger-Kawahara and Horwitz also reported that activation of complement in NHS by viable legionellae occurred exclusively via the alternative pathway. On the basis of their findings, MOMP is thought to activate the alternative pathway, bind C3, and promote the uptake of *L. pneumophila* by human monocytes and macrophages. However, in contrast with the findings of Bellinger-Kawahara and Horwitz, others have noted that incubation of legionellae in NHS resulted in activation of the classical pathway with no detectable activation of the alternative pathway (22, 31). Moreover, Verbrugh et al. (31) and Husmann and Johnson (16) showed that the classical pathway appears to play an important role in the uptake of legionellae by mononuclear phagocytes.

In the present study, we sought to clarify the roles of the classical and alternative pathways in the activation of complement by *L. pneumophila.*

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***L. pneumophila* activates both the classical pathway and the alternative pathway in NHS.** In the report by Bellinger-Kawahara and Horwitz, the ability of strain Philadelphia 1 to bind C3 in NHS treated with EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] and Mg^{2+} suggested that activation of complement in NHS by *L. pneumophila* occurred via the alternative pathway (4). EGTA treatment inhibits the classical pathway but leaves the alternative pathway intact and reactive (8, 17). It is important to note, however, that although EGTA-treated serum is frequently used to measure the involvement of the alternative pathway in C activation, EGTA-chelated serum does not always yield clear-cut results (17). Therefore, to more clearly define the roles of the classical and alternative pathways in the activation of complement by *L. pneumophila*, we assessed the ability of whole cells of strain Philadelphia 1 to bind C3 during incubation in C1q-depleted serum (which is devoid of classical pathway activity) or factor B-depleted serum (which lacks a functional alternative pathway). C3 binding to strain Philadelphia 1 was measured by an enzyme-linked immunosorbent assay (ELISA) similar to the one described previously by Bellinger-Kawahara and Horwitz (4).

In these experiments, 10^8 late-logarithmic-phase cells of strain Philadelphia 1, harvested from ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid]-buffered charcoal-yeast extract plates, were incubated for 1 h at 37°C in pooled NHS (PNHS) (prepared in our laboratory as previously described [23]) treated with 20 mM EGTA plus 10 mM $MgCl_2$ or NHS depleted of either C1q or factor B. Bacteria were also incubated in C1q-depleted NHS reconstituted with purified human C1q- and factor B-depleted NHS that contained added purified human factor B. C3 binding to legionellae incubated in each of the previously mentioned sera treated with 20 mM EDTA (which inhibits both complement pathways) was also measured. Complement component-depleted NHS and purified human C1q and factor B were obtained from Sigma Chemical Co., St. Louis, Mo., or Quidel, San Diego, Calif. Analysis of commercial preparations of purified C1q and factor B by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoelectrophoresis confirmed that each preparation contained a single protein species that corresponded to either C1q or factor B. Functional hemolytic assays confirmed that C1q-

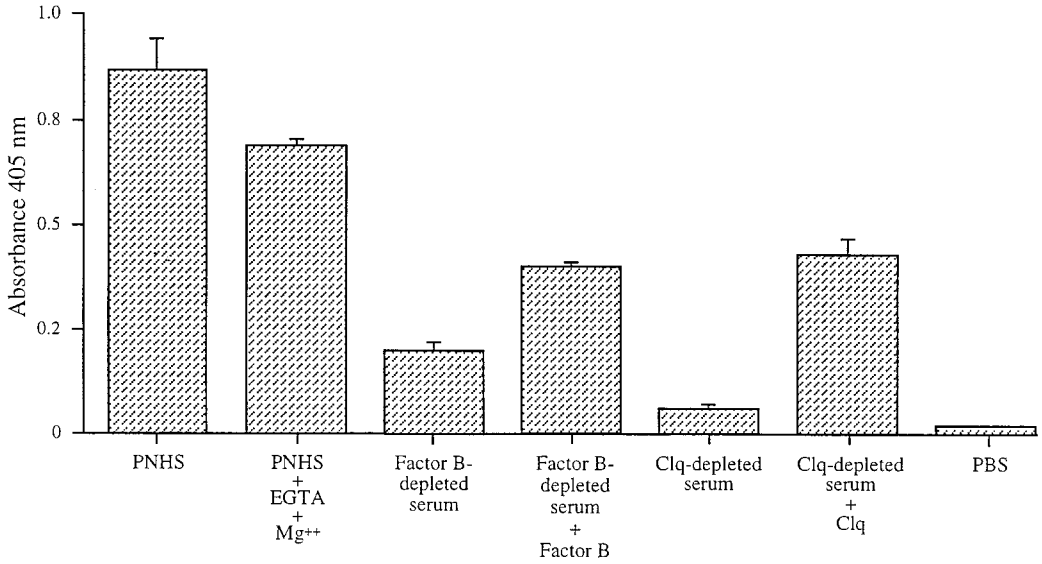

FIG. 1. *L. pneumophila* Philadelphia 1 binds C3 via activation of the classical and alternative pathways of complement. C3 binding by bacteria incubated in each of the indicated sera was measured by an ELISA as described in the text. Reconstituted factor B-depleted serum contained 250 μg of purified factor B per ml, whereas reconstituted C1q-depleted serum contained 150 μg of purified human C1q per ml. A_{405} was calculated by subtracting the A_{405} of legionellae incubated in EDTA-chelated serum from the A_{405} of cells incubated in the corresponding serum not treated with EDTA.

deficient NHS was devoid of classical activity but contained a functional alternative pathway whereas factor B-depleted NHS lacked a functional alternative pathway but retained classical pathway activity (22).

After incubation in the various sera, bacteria were collected by centrifugation, washed several times with phosphate-buffered saline (PBS), and resuspended to a concentration of 10^8/ml. Then, 0.1-ml samples of the bacterial suspensions were dispensed in triplicate to wells of 96-well tissue culture plates (Falcon, Lincoln Park, N.J.) and allowed to evaporate to dryness by overnight incubation at 37°C. The wells were treated with PBS containing 3% bovine serum albumin (BSA) for 2 h at ambient temperature to block nonspecific protein binding. The blocked wells were washed several times with PBS, incubated with peroxidase-conjugated goat anti-human C3 immunoglobulin G (IgG) (Cappel, West Chester, Pa.) for 2 h at room temperature, washed again, and incubated with peroxidase substrate (23). Color was allowed to develop for 20 min, and A_{405} was measured immediately. Final A_{405} values were calculated by subtracting the A_{405} of legionellae incubated in EDTA-chelated NHS from the A_{405} of cells incubated in the corresponding serum devoid of EDTA. This was done to control for nonspecific binding of antibodies to C3 to opsonized Philadelphia 1 cells.

Bacteria incubated in PNHS bound substantial amounts of C3 (Fig. 1). In agreement with the findings of Bellinger-Kawahara and Horwitz, Philadelphia 1 cells incubated in PNHS treated with EGTA and Mg^{2+} retained the ability to bind C3. However, in contrast to their results, we consistently observed a reduction in C3 binding by bacteria incubated in EGTA-treated PNHS compared with untreated PNHS (Fig. 1).

Philadelphia 1 cells incubated in factor B-depleted serum bound small amounts of C3 (Fig. 1). C3 binding by these cells increased twofold after the addition of physiological amounts of purified factor B (250 μg/ml) to the factor B-depleted serum (Fig. 1). Together, these data indicated that the alternative pathway played a role in the activation of complement by strain Philadelphia 1. However, it is important to note that, in the absence of a functional alternative pathway, the bacteria retained the ability to bind C3. This provided evidence that the classical pathway contributed to complement activation during incubation of legionellae in NHS.

Philadelphia 1 cells incubated in C1q-depleted serum bound very little C3 (Fig. 1). This result was somewhat surprising, since we had previously demonstrated that the alternative pathway is involved in complement activation by *L. pneumophila*. It is likely that the small amount of C3 bound by legionellae incubated in C1q-depleted NHS resulted from a diminution in alternative pathway activity following the removal of C1q from this serum (24). Nevertheless, the addition of physiological amounts of purified C1q (150 μg/ml) to C1q-depleted NHS resulted in an almost eightfold increase in the amount of C3 bound by Philadelphia 1 cells (Fig. 1). This result clearly demonstrated that the classical pathway played a role in the activation of complement by strain Philadelphia 1 during incubation in NHS.

Antibody-independent binding of C1q to viable legionellae. Activation of the classical pathway can occur via antibody-dependent or -independent mechanisms. Antibody-dependent activation of the classical pathway is initiated via binding of C1q to the Fc portion of immunoglobulins bound to the complement activator (24, 32). In contrast, antibody-independent activation occurs by the direct binding of C1q to the activator (20, 21). To determine which of these two mechanisms contributed to the activation of the classical pathway, we measured the amount of C1q bound by legionellae incubated in PNHS or immunoglobulin-deficient serum.

Immunoglobulin-deficient serum (kindly provided by Charles Mitchell, University of Miami School of Medicine, Miami, Fla.) was obtained from an agammaglobulinemic (AG) patient with Bruton's disease. Functional hemolytic assays indicated that the classical and alternative pathways were intact and reactive in the AG serum and that the same serum contained normal levels of C1, C2, C3, and C4 (22). Three serogroup 1 strains, Philadelphia 1, Knoxville 1, and Ver5, and one serogroup 3 strain, Bloomington 2, were used in these experiments. These strains of *L. pneumophila* were selected because

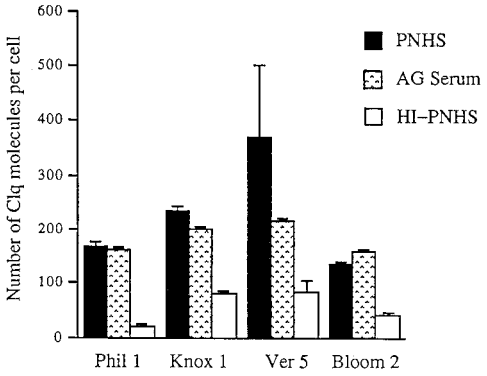

FIG. 2. Binding of human C1q to different strains of *L. pneumophila*. Each *L. pneumophila* strain (5×10^8 cells) was incubated in PNHS, HI-PNHS, or AG serum for 1 h at 37°C. After incubation, bound C1q was detected with ^{125}I-labeled antibodies to C1q, and the number of C1q molecules per cell was calculated as described in the text. Abbreviations: Phil 1, Philadelphia 1; Knox 1, Knoxville 1; Bloom 2, Bloomington 2.

they had previously been shown to activate complement and bind substantial amounts of C3 (4, 29).

To measure the binding of C1q to viable legionellae, late-logarithmic to early-stationary-phase cells of *L. pneumophila* were harvested after 3 days of growth at 37°C on ACES-buffered charcoal-yeast extract agar plates; washed several times in glucose-gelatin-Veronal buffer supplemented with 0.5 mM $MgCl_2$, 0.15 M $CaCl_2$, and 75 mM NaCl (pH 7.5) ($DGVB^{2+}$); and adjusted to a concentration of 5×10^8/ml. One-milliliter portions of the bacterial suspension were added to four sterile 1.9-ml Microfuge tubes (Baxter Scientific, Miami, Fla.) and centrifuged at 8,000 rpm for 6 min in a Savant Microfuge (Savant Instruments Inc., Farmingdale, N.Y.). The pellets were suspended in 100 μl of PNHS, AG serum, heat-inactivated (56°C for 30 min) PNHS (HI-PNHS), or $DGVB^{2+}$ and incubated with rotation for 1 h at 37°C. The cells were recovered by centrifugation, washed twice with $DGVB^{2+}$ (1.0 ml per wash), and then incubated with rotation for 1 h at 25°C in 250 μl of $DGVB^{2+}$ that contained 2×10^5 cpm of ^{125}I-labeled goat anti-human C1q IgG. C1q antibodies were obtained from Cappel and labeled with ^{125}I by the Iodobead method outlined by the manufacturer (Pierce Chemical Co., Rockford, Ill.). Cells were recovered by centrifugation and washed three times with 1.0 ml of $DGVB^{2+}$ to remove nonspecifically bound radioactivity. After the final wash, the tips of the Microfuge tubes were cut off, and the amount of radioactivity associated with each bacterial pellet was measured in a gamma counter (Pharmacia, Gaithersburg, Md.). The amount of ^{125}I-labeled C1q antibody bound by bacteria incubated in PNHS, AG serum, or HI-PNHS was determined after subtracting the radioactivity bound by bacteria incubated in $DGVB^{2+}$ (which represented nonspecific binding of the ^{125}I-labeled C1q antibodies). The number of C1q molecules per bacterium was calculated by assuming that the number is the same as the number of molecules of ^{125}I-IgG bound per cell during incubation in the antiserum. The calculations were based on a molecular mass for goat IgG of 150,000 Da.

The results of these experiments indicated that the serogroup 3 strain and all of the serogroup 1 strains bound C1q after incubation in PNHS and AG serum (Fig. 2). C1q binding was greatly diminished when bacteria were incubated in HI-PNHS. This was expected because C1q is heat labile (24). Of interest, all of the strains bound similar amounts of C1q, ca. 170 to 230 molecules per cell. Moreover, there was no difference in the amount of C1q bound by each of the strains after incubation in PNHS or AG serum. These results, along with the C3 binding data presented above, suggested that *L. pneumophila* activates the classical pathway in NHS via the direct binding of C1q to its cell surface.

Isolated MOMP binds C1q. As mentioned previously, MOMP can activate complement and bind C3 (4). Of interest, outer membrane proteins and porins from several gram-negative bacteria including *Escherichia coli*, *Salmonella typhimurium*, *Salmonella minnesota*, and *Klebsiella pneumoniae* can bind human C1q directly (1, 18, 21, 28). To determine whether MOMP can act as a C1q acceptor on the *L. pneumophila* surface, we tested the interaction between human C1q and isolated MOMP preparations. In these experiments, various amounts of MOMP, which was isolated and purified from strain Philadelphia 1 by the methods of Ehret and Ruckdeschel (6), or BSA were blotted to nitrocellulose by using a slot blot apparatus (Bethesda Research Laboratories, Bethesda, Md.). These blots were incubated in Tris-buffered saline (15 mM Tris-HCl, 150 mM NaCl [pH 8.0]) containing 3% BSA (TBS-BSA) for 2 h at 25°C, washed several times with TBS, and incubated in 5 ml of TBS-BSA containing 5 μg of purified human C1q per ml for 15 min at room temperature with gentle shaking. The C1q used in these experiments was purified from NHS as described by Zhang et al. (33) and did not contain any detectable IgG or IgM antibodies (22). After incubation, the blots were washed once with TBS, once with TBS plus 0.1% Nonidet P-40 (Sigma) (TBS-NP40), and once with TBS to remove any nonspecifically bound C1q. The blots were then incubated with 5×10^4 cpm of ^{125}I-labeled goat anti-human C1q IgG (0.28 μCi/μg) per ml in 5 ml of TBS-BSA for 1 h at 25°C with gentle shaking. After incubation, the blots were washed three times with TBS and TBS-NP40 as described above, dried, and subjected to autoradiography using X-Omat AR film (Kodak, Rochester, N.Y.).

The results from these experiments indicated that purified MOMP bound C1q whereas BSA did not (Fig. 3A). Similar results were obtained when blots containing purified MOMP

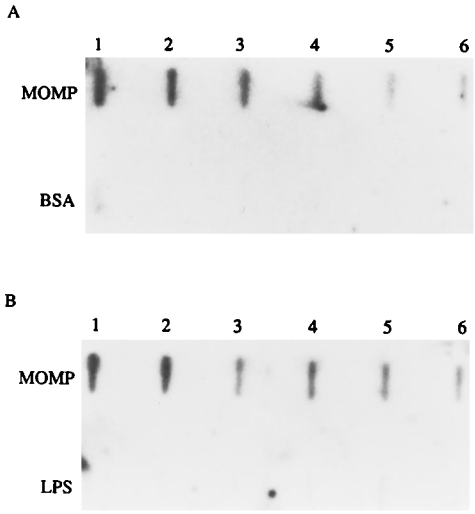

FIG. 3. Binding of purified human C1q to MOMP. Equal amounts of MOMP, LPS, and BSA were slot blotted onto nitrocellulose, incubated with human C1q (5 μg/ml) for 15 min at 25°C, and probed with ^{125}I-labeled antibodies to C1q as described in the text. (A) Amount of MOMP or BSA (in micrograms): lane 1, 50; lane 2, 25; lane 3, 12.5; lane 4, 6.25; lane 5, 3.12; lane 6, 1.56. (B) Amount of MOMP or LPS (in micrograms): lane 1, 25; lane 2, 12.5; lane 3, 6.25; lane 4, 3.12; lane 5, 1.56; lane 6, 0.75.

or BSA were incubated in immunoglobulin-deficient AG serum (22).

It has been reported that C1q can bind to the lipid A portion of some lipopolysaccharides (LPS) (19, 23). We determined by ELISA that our MOMP preparations contained ca. 0.3 μg of serogroup 1 LPS per 100 μg of MOMP. To determine whether the small amount of LPS contained in our MOMP preparations was responsible for C1q binding, blots containing equal amounts of MOMP and serogroup 1 LPS, prepared as described by Mintz et al. (23), were incubated with purified human C1q and then probed with ^{125}I-labeled antibodies to C1q as described above. In contrast to MOMP, purified serogroup 1 LPS failed to bind detectable amounts of C1q (Fig. 3B).

The results of experiments outlined above suggested that MOMP may be the ligand responsible for the antibody-independent binding of C1q to the *L. pneumophila* cell surface. However, unlike two other complement components, i.e., C3b and C4b, C1q binds to activators via noncovalent interactions (24). This prevented us from using cell fraction techniques coupled with Western blot (immunoblot) analysis to determine whether C1q could bind directly to MOMP found on the *L. pneumophila* cell surface. Instead, we reasoned, if C1q can bind to MOMP on the *L. pneumophila* surface, then bound C1q may sterically hinder the binding of MOMP antibodies to legionellae after incubation in NHS. To test this possibility, we performed the following experiments.

Late-logarithmic-phase cells (5×10^8) of strain Philadelphia 1 were incubated in AG serum, HI-AG, and $DGVB^{2+}$ (all of which contained 20 mM EDTA) for 1 h at 37°C. HI-AG serum was included in these experiments to control for nonspecific binding of radiolabeled antibodies to opsonized legionellae. It was necessary to add EDTA to serum preparations in these experiments because a previous report (4) indicated that MOMP can bind activated C3 during incubation in NHS. EDTA inhibits the classical and alternative pathways and prevents C3 activation but does not interfere with C1q binding (24, 33). After incubation, 150,000 cpm of ^{125}I-labeled polyclonal antibodies to MOMP (prepared in this laboratory) were added to duplicate samples of each bacterial suspension, the samples were incubated for 1 h at 25°C, and the amount of cell-associated radioactivity was determined as described previously. The amount of C1q bound by opsonized legionellae in these experiments was determined by adding ^{125}I-labeled C1q antibodies to bacterial suspensions that were not treated with radiolabeled MOMP antibodies. The number of C1q molecules bound per cell was calculated as described above.

The amount of C1q bound by Philadelphia 1 cells was essentially the same as that observed in previous experiments (Fig. 2). However, there was no detectable decrease in the amount of MOMP antibody bound by legionellae incubated in AG serum compared with cells incubated in buffer or HI-AG (22). This indicated that the presence of C1q on the Philadelphia 1 cell surface did not interfere with the binding of antibodies directed against MOMP.

The results of the present study, for NHS depleted of either classical pathway activity or alternative pathway activity, indicated that both pathways participate in the activation of complement during incubation of strain Philadelphia 1 in NHS (Fig. 1). As mentioned above, Bellinger-Kawahara and Horwitz found that incubation of Philadelphia 1 in NHS resulted in activation of the alternative pathway, whereas the work of Verbrugh et al. suggested that activation of complement occurred via the classical pathway. Differences in the assay systems used to measure complement activation in these studies may partially account for the seemingly disparate results obtained by these two groups. Nevertheless, it is apparent from our study that both pathways contributed to the activation of complement by *L. pneumophila*.

The ability of isolated *L. pneumophila* MOMP (which was previously determined to be a porin [9]) to bind C1q independently of antibody (Fig. 3) is consistent with the results of several investigators who demonstrated that porins from other gram-negative bacteria, including *S. minnesota* (18, 28), *S. typhimurium* (20), and *K. pneumoniae* (1), can directly bind C1q. Moreover, the direct binding of C1q by bacterial porins can result in activation of the classical pathway during incubation of purified porins in NHS (1, 10). In agreement with these observations, we recently determined, using functional hemolytic assays (23), that purified MOMP preparations can activate the classical pathway in immunoglobulin-deficient AG serum (22).

The molecular basis of C1q binding to bacterial porins is not known. It has been suggested that the negative charge of many porins (pI 4.5 to 5.0) may explain binding of the positively charged C1q (pI > 9.0). In agreement with this idea, Hoffman and coworkers (12, 13) determined that *L. pneumophila* MOMP is a negatively charged molecule with a pI of 4.59. Of interest, Albertí et al. (2) recently localized a putative C1q binding site on the outer membrane protein K36 porin of *K. pneumoniae* that resembles the binding site known to mediate the interaction between C1q and the Fc portion of IgG antibodies. These authors suggested that a common C1q binding motif may be present in bacterial porins since all porins tested to date bind C1q. The gene encoding *L. pneumophila* MOMP has been cloned and sequenced (12). In light of the findings of Albertí et al. and our results, which showed that *L. pneumophila* MOMP can bind C1q independently of antibody, we are currently examining the deduced amino acid sequence of MOMP for possible C1q binding domains.

Since incubation of *L. pneumophila* in NHS activated the classical pathway (Fig. 1), it was not surprising that incubation of viable, intact *L. pneumophila* cells in either NHS or AG resulted in binding of C1q to the organism's cell surface (Fig. 2). The binding of equivalent amounts of C1q by legionellae incubated in NHS or AG suggested that binding of C1q to the *L. pneumophila* cell surface was antibody independent. On the basis of the ability of purified MOMP to bind C1q, MOMP may be the C1q binding ligand during incubation of whole legionellae in NHS. However, the results of experiments to determine whether MOMP can act as a C1q acceptor on the *L. pneumophila* cell surface were inconclusive. As mentioned previously, preincubation of strain Philadelphia 1 in EDTA-treated PNHS and AG serum resulted in deposition of C1q on the *L. pneumophila* surface but failed to interfere with the binding of polyclonal antibodies directed against MOMP. One explanation for these results is that only a small number of MOMP molecules on the *L. pneumophila* cell surface actually bind C1q during incubation of legionellae in serum. If this is the case, then a detectable decrease in binding of polyclonal MOMP antiserum would not be expected since the majority of MOMP molecules could still react with the antibodies to MOMP. The binding of relatively small amounts of C1q by the strains of *L. pneumophila* tested in our study would tend to support this idea. Additional experiments using radiolabeled C1q and cross-linking agents may define the mechanism by which MOMP binds C1q during incubation of legionellae in NHS.

Horwitz and coworkers (4, 26) have proposed that opsonic C3 (as C3b) and corresponding phagocyte complement receptors CR1 and CR3 promote the uptake of *L. pneumophila* by mononuclear phagocytes. However, on the basis of our findings, it is tempting to speculate that C1q found on the *L.*

pneumophila surface may facilitate uptake via C1q receptors present on mononuclear phagocytes (11, 30). In support of this idea, Alvarez-Dominguez et al. (3) recently demonstrated that C1q found on the surface of *Listeria monocytogenes* promoted the phagocytosis of this organism via C1q receptors found on murine macrophages. Future studies are planned to determine whether C1q and corresponding C1q receptors plays a similar role in the uptake of *L. pneumophila* by human mononuclear phagocytes.

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