Complement Component C3 Fixes Selectively to the Major Outer Membrane Protein (MOMP) of *Legionella pneumophila* and Mediates Phagocytosis of Liposome-MOMP Complexes by Human Monocytes

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Summary

Legionella pneumophila is a facultative intracellular bacterial pathogen that parasitizes human monocytes and alveolar macrophages. Previous studies from this laboratory have shown that monocyte complement receptors CR1 and CR3 and complement component C3 in serum mediate *L. pneumophila* phagocytosis. In this study, we have explored C3 fixation to *L. pneumophila*. We developed a whole-cell enzyme-linked immunosorbent assay (ELISA) to measure C3 fixation to the bacterial surface. By this assay, C3 fixes to *L. pneumophila* that are opsonized in fresh nonimmune serum, and C3 fixation takes place via the alternative pathway of complement activation. Immunoblot analysis of opsonized *L. pneumophila* indicated that C3 fixes selectively to specific acceptor molecules of *L. pneumophila*. Consistent with this, when nitrocellulose blots of whole *L. pneumophila* or bacterial components are incubated in fresh nonimmune serum, C3 fixes exclusively to the major outer membrane protein (MOMP) of *L. pneumophila*, a porin; C3 does not fix to *L. pneumophila* LPS on these blots. To further explore the role of MOMP in C3 fixation and phagocytosis, we reconstituted purified MOMP into liposomes. By the ELISA, MOMP-liposomes, but not plain liposomes lacking MOMP, avidly fix C3. Consistent with a dominant role for MOMP in C3 fixation, MOMP-liposomes form a C3 complex of the same apparent molecular weight as whole *L. pneumophila* in nonimmune serum. Opsonized radioiodinated MOMP-liposomes avidly adhere to monocytes, and adherence is dose dependent upon serum. By electron microscopy, opsonized MOMP-liposomes are efficiently phagocytized by human monocytes, and phagocytosis takes place by a conventional appearing form of phagocytosis. This study demonstrates that C3 fixes selectively to the MOMP of *L. pneumophila*, and that, in the presence of nonimmune serum, MOMP can mediate phagocytosis of liposomes and, potentially, phagocytosis of intact *L. pneumophila* by human monocytes.

Legionella pneumophila, the etiologic agent of Legionnaires' disease, is a facultative intracellular bacterial pathogen that parasitizes human monocytes and alveolar macrophages (1, 2). The bacterium gains access to these mononuclear phagocytes by phagocytosis. In the case of *L. pneumophila*, the ingestion process has an unusual morphology and has been termed "coiling phagocytosis" (3). Once intracellular, the bacterium resides in a ribosome-lined phagosome of relatively high pH that does not fuse with lysosomes (4–6). *L. pneumophila* multiplies in this specialized phagosome and ultimately destroys the host cell.

Previous studies from this laboratory have demonstrated that complement receptors CR1 and CR3 on human monocytes mediate phagocytosis of *L. pneumophila* (7). These receptors recognize fragments of complement component C3; CR1 is the primary receptor for C3b, and CR3 is the primary receptor for iC3b. mAbs against these receptors inhibit phagocytosis of *L. pneumophila* and, consequently, intracellular multiplication of the bacteria in human monocytes. Although complement receptors may recognize molecules other than complement components, previous studies have indicated that complement component C3 mediates phagocytosis of *L. pneumophila*. Consistent with this, phagocytosis of *L. pneumophila* is serum dependent, mediated by a component in nonimmune serum capable of fixing to the bacterium, and markedly reduced in heat-inactivated serum (7). However, previous studies using an immunofluorescence assay did not detect C3 on the surface of *L. pneumophila* in nonimmune serum (8).

In this study, we first sought to demonstrate that C3 is fixed to *L. pneumophila*. To achieve this goal, we developed a highly sensitive whole cell ELISA to detect C3 on the *L.*

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pneumophila surface. Using this assay, we shall demonstrate that C3 is fixed to the bacterium and that fixation takes place by the alternative pathway of complement activation.

We also sought in this study to identify acceptor molecule(s) for C3 on the *L. pneumophila* surface. Although, in theory, any surface protein or LPS molecule with an accessible COOH or NH_2 group might serve this purpose, we found that C3 is fixed predominantly to a single molecule on *L. pneumophila*. We shall demonstrate that C3 in nonimmune serum is fixed selectively to the major outer membrane protein (MOMP)[1] of the bacterium, a porin. We shall also show that liposomes with purified MOMP incorporated into their membranes readily fix C3, and when so opsonized, the liposomes are efficiently phagocytized by human monocytes.

Materials and Methods

Reagents. Goat anti-human C3 (Atlantic Antibodies, Scarborough, ME); alkaline phosphatase (AP)-conjugated rabbit anti-goat IgG and AP-conjugated goat anti-rabbit IgG (Cappel Organon Teknika Corp., Westchester, PA); horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, HRP-conjugated rabbit anti-goat IgG, and HRP-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA); mouse monoclonal anti-human iC3b neoantigen (Cytotech, San Diego, CA); chicken anti-human C3 (Accurate Chemical & Scientific Corp., Westbury, NY); HRP-conjugated goat anti-chicken IgG (Bethyl Labs Inc., Montgomery, TX); nitrocellulose blotting paper (Schleicher & Schuell, Inc., Keene, NH); and diphytanoylphosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) (Avanti Polar Lipids, Inc., Birmingham, AL) were purchased. All other reagents were obtained from commercial sources and were reagent-grade quality, except as noted.

Bacteria. *L. pneumophila*, Philadelphia 1 strain, was grown in embryonated hens' eggs, harvested, tested for viability and contaminants, and stored at −70°C, as described (1). For experimental use, egg yolk–grown *L. pneumophila* was cultured one time only on charcoal yeast extract agar, as described (1). Colonies were removed from the surface of several agar plates in sterile PBS and harvested by centrifugation at 4°C at 10,000 *g*. The harvested bacteria were washed twice more, resuspended in PBS, quick-frozen in a dry ice-ethanol bath, and stored at −70°C.

L. pneumophila Membranes, MOMP, and LPS. *L. pneumophila* membranes were prepared by making and lysing spheroplasts, and MOMP and LPS were purified from the membranes as described (9, 10).

Antisera. Antisera against the MOMP and LPS of *L. pneumophila* were obtained by immunizing 2-kg New Zealand White rabbits subcutaneously with purified MOMP or purified LPS. Rabbits were initially injected with 100 μg MOMP or 50 μg LPS mixed 1:1 with CFA, and then boosted 2 and 4 wk later with 100 μg MOMP or 50 μg LPS mixed 1:1 with IFA.

Serum. Fresh human serum was obtained from normal nonimmune donors and stored at −70°C until use. Serum aliquots were thawed on ice and used immediately, or were heat inactivated at 56°C for 30 min before use.

ELISA for C3 Fixation to L. pneumophila and Liposomes. *L. pneumophila* (10^8 bacteria/ml) were incubated for 60 min at 37°C in fresh nonimmune human serum, fresh serum containing 10 mM EDTA, fresh serum containing 10 mM EGTA and 7 mM $MgCl_2$, heat-inactivated serum, or PBS. Bacteria were harvested by centrifugation, washed twice in PBS, and resuspended in PBS at a final concentration of 10^8 bacteria/ml. 0.1-ml aliquots of the bacterial suspension were dispensed in triplicate to wells of a 96-well tissue culture plate and allowed to evaporate to dryness under a laminar flow hood. Nonspecific protein binding sites were then blocked by the addition of PBS containing 3% OVA for a minimum of 6 h. The blocked wells were washed three times with PBS, incubated with primary antibody for 1 h at room temperature, washed three times more in PBS, incubated with AP-conjugated secondary antibody for 1 h at room temperature, washed again, and incubated with phosphatase substrate. The color was allowed to develop for 30 min, and then the reaction was terminated by the addition of NaOH to a final concentration of 0.2 N. Absorbance at 405 nm was measured immediately.

Liposomes with and without MOMP incorporated in their membranes were incubated for 60 min at 37°C in 10% fresh serum or in 10% fresh serum containing 10 mM EDTA. The liposomes were harvested by ultracentrifugation, washed 3 times with PBS, and resuspended in PBS. One-tenth ml aliquots (1.5-1.8 μg total phospholipid) were dispensed in triplicate wells, evaporated to dryness, and assayed for C3 as above.

SDS-PAGE. SDS-PAGE was performed according to Laemmli (11) as modified by Ames (12). Separating gels contained 4 M urea and either 7.5% or 14% polyacrylamide. Samples were solubilized in SDS-PAGE sample buffer containing 62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 2 mM EDTA, 5% 2-ME, and either bromphenol blue or pyronin Y as a tracking dye. Molecular weight standards (Sigma Chemical Co., St. Louis, MO) were as follows: BSA, 66 kD; OVA, 45 kD; glutaraldehyde-6-phosphate dehydrogenase, 36 kD; carbonic anhydrase, 29 kD; trypsinogen, 24 kD; and α-lactalbumin, 14.2 kD. Prestained molecular weight standards (Bio-Rad Laboratories), calibrated before use, were as follows: prestained carbonic anhydrase, 37 kD; prestained OVA, 52 kD; prestained BSA, 87 kD.

Immunoblot Analysis of C3 Fixation to L. pneumophila and Liposomes. *L. pneumophila* (3.6×10^8 bacteria) or liposomes with or without MOMP (5 μg phospholipid) were opsonized by incubation in serum at 37°C. In some cases, PMSF was added after incubation to prevent proteolytic breakdown of C3. The bacteria and liposomes were vigorously washed in PBS and subjected to SDS-PAGE. The separated proteins were then electroblotted to nitrocellulose paper for 12–18 h at 0.25 A at 4°C. The blots were first incubated in PBS containing 1% BSA at 4°C overnight to block nonspecific protein binding sites, and then incubated at 4°C for 12–18 h with primary antibody diluted in PBS containing 1% BSA, 0.02% SDS, and 0.1% Triton X-100. The blots were washed three times for 10 min each in PBS containing 0.2% BSA, 0.02% SDS, and 0.1% Triton X-100, and then incubated at 4°C for 18 h with enzyme-conjugated secondary antibody, diluted in PBS containing 1% BSA, 0.02% SDS, and 0.1% Triton X-100. The blots were washed three times as above and substrate was added.

In some experiments, radiolabeled C3, kindly provided by Jo Ellen Schweinle and Keith Joiner (Yale University), was added to serum during the opsonization step, and autoradiographs of the SDS-PAGE gel were obtained.

Analysis of C3 Fixation to Immunoblotted L. pneumophila Components. Triplicate sets of whole *L. pneumophila*, *L. pneumophila* membranes, isolated MOMP, and isolated LPS were subjected to SDS-PAGE (14% polyacrylamide-4 M urea gels) and the separated molecules immunoblotted to nitrocellulose paper. The blots were incubated overnight at 4°C in PBS containing 1% BSA to block

[1] *Abbreviations used in this paper:* AP, alkaline phosphatase; HRP, horseradish peroxidase; MOMP, major outer membrane protein; PC, diphytanoylphosphatidylcholine; PE, egg phosphatidylethanolamine.

nonspecific protein binding sites. One set of blots was incubated in 10% fresh human serum in PBS for 60 min at 37°C, washed with PBS three times for 15 min each to remove unbound serum components, and probed with a primary antibody against C3. The other two sets of blots were not incubated in serum; one of these was probed with a primary antibody against MOMP, and the other was probed with a primary antibody against LPS. HRP-conjugated secondary antibodies were added and the blots developed as described in the above assay.

Construction of Liposomes with and without MOMP. Liposomes were constructed according to the cholate dilution procedure of Racker et al. (13). PC and PE were dissolved separately in chloroform/methanol (2:1 [vol/vol]) and mixed at a ratio of four parts PC to one part PE (wt/wt). The mixed lipids (6.5 mg total phospholipid) were dried under N_2, diluted in 195 μl PBS, and sonicated to clarity at 4°C. When liposomes were constructed with MOMP, the sonicate was mixed with MOMP at a ratio of 50:1 (wt/wt) phospholipid/protein. Cholate was added to a final concentration of 0.1%, and the mixture incubated at 4°C for 30 min. The liposomes were diluted to 25 times the sample volume, incubated for 10 min at room temperature, pelleted at 192,000 *g*, washed once, and resuspended in PBS.

Total phospholipid in liposomes was quantitated by the method of Raheja et al. (14) using a 4:1 mixture of PC and PE as a standard. Duplicate liposome samples were extracted twice with chloroform/methanol, (2:1 [vol/vol]). The organic phases were pooled and evaporated to dryness in acid-cleaned glass tubes for use in the assay.

Total protein in liposomes was quantitated by the method of Lowry et al. (15) using BSA as a standard. The aqueous phases from extracted liposomes were used to measure incorporated protein.

Preparation and Opsonization of Radioiodinated MOMP-Liposomes. In studies on adherence of MOMP-liposomes to human monocytes, liposomes were constructed as above, but with radioiodinated MOMP. Purified MOMP was iodinated with ^{125}I by coupling with Bolton-Hunter reagent (*N*-succinimidyl 3-(4-hydroxy, 5-[^{125}I]-iodophenyl)propionate). MOMP (100 μg) in 0.1 M borate buffer, pH 8.5, was reacted at 0°C for 30 min with 250 μCi Bolton-Hunter reagent that had been dried onto the surface of a glass tube. The reaction was terminated with the addition of 0.1 M borate buffer, pH 7.5, containing 0.2 M glycine. The iodinated MOMP was precipitated from 80% ethanol at −70°C, harvested by centrifugation, and resuspended at 1 μg/μl in PBS. Liposomes containing radioiodinated MOMP (sp act 3–8 × 10^4 cpm/μg) were opsonized for 60 min at 37°C in varying concentrations of fresh serum, harvested by ultracentrifugation, and washed twice in PBS.

Assay for Adherence of Opsonized MOMP-Liposomes to Human Monocytes. Human mononuclear cells were isolated from fresh heparinized blood as described previously (1). Mononuclear cells (3 × 10^6/coverslip) in RPMI 1640 containing 25 mM Hepes and 20% autologous serum were applied to sterile coverslips in 24-well tissue culture plates, and incubated in 5% CO_2 at 37°C for 60 min. The adherent monocytes were washed several times with RPMI containing 25 mM Hepes to remove nonadherent cells and serum components and then incubated under serumless conditions with opsonized ^{125}I-MOMP-containing liposomes (58,000 cpm, 2 μg phospholipid) at 37°C in 5% CO_2 under rotation at 100 rpm. After 60 min, the monocytes were washed thoroughly with RPMI to remove unbound liposomes, and monocyte-associated counts per minute were determined. All experiments were performed in triplicate.

Electron Microscopic Study of Phagocytosis of Liposome-MOMP-C3 Complexes. Liposomes containing MOMP (10 μg phospholipid) were incubated in 10% fresh serum for 60 min at 37°C, harvested by ultracentrifugation, washed thoroughly, and suspended in PBS. Mononuclear cells (7.2 × 10^5) were gently pelleted and chilled to 0°C. Opsonized liposomes, also at 0°C, were layered over the mononuclear cells and pelleted at 325,000 *g* for 15 min. The samples were shifted to 37°C to allow phagocytosis to proceed, and then fixed for electron microscopy after 3 or 6 min. Controls included plain liposomes incubated with serum under the same conditions and MOMP-liposomes incubated in serum containing EDTA. Samples were fixed for electron microscopy with a solution consisting of 1% OsO_4 (two parts) and 2.5% glutaraldehyde (one part) in 0.1 M cacodylate buffer, pH 7.4, stained with 0.25% uranyl acetate in 0.1 M sodium acetate buffer, pH 6.3, and dehydrated with ethanol. The samples were infiltrated and embedded in Spur medium, sectioned, and stained with lead citrate and uranyl acetate. Sections were examined with an electron microscope (JEM-100 CX; JEOL, Ltd., Tokyo, Japan).

Results

L. pneumophila Fixes Complement Component C3 to its Surface. To detect C3 on the surface of *L. pneumophila*, we developed a sensitive whole bacterial cell ELISA. To minimize alterations of the bacterial cell surface, we applied intact, opsonized bacteria to microtiter wells in solution and then allowed the solution to evaporate to dryness. We thereby avoided a harsh chemical treatment that might denature or oxidize delicate surface structures.

Bacteria opsonized in fresh nonimmune serum readily fixed C3 (Fig. 1). In contrast, bacteria incubated in buffer only did not demonstrate the presence of C3, and bacteria opsonized in heat-inactivated serum exhibited only a small, but reproducible amount of C3 fixation. C3 associated with the bacteria was not removed by washing with detergents (2% Triton X-100, Zwittergent 3-14, or SDS). The amount of C3 fixed to bacteria incubated in fresh serum was proportional to the the serum concentration (data not shown). Bacteria used in these assays were freshly thawed, viable organisms. Interest-

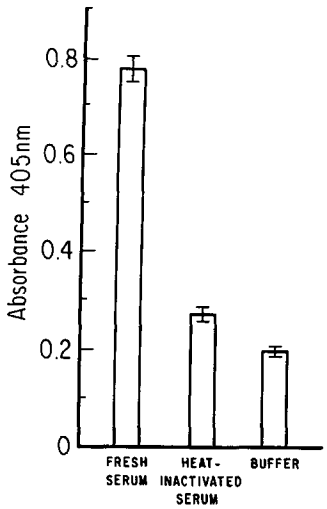

Figure 1. *L. pneumophila* fixes complement component C3 to its surface. *L. pneumophila* was incubated for 60 min at 37°C in fresh, nonimmune human serum, in heat-inactivated serum, or in PBS. The bacteria were washed and used as antigen in an ELISA for C3 as described in the text. Data are the mean ± SD of triplicate measurements.

ingly, heat-killed and formalin-killed *L. pneumophila* also fixed C3 in the same way (data not shown).

L. pneumophila Fixes Complement Component C3 by the Alternative Pathway of Complement Activation. To determine if C3 fixation occurs by the classical or alternative pathway of complement activation, we assayed C3 fixation to *L. pneumophila* that were incubated in fresh serum alone, in fresh serum containing 10 mM EGTA and 7 mM $MgCl_2$ (conditions under which classical pathway activation is abolished but alternative pathway activation proceeds), in fresh serum containing 10 mM EDTA (conditions under which both pathways are abolished), or in buffer only. Bacteria opsonized in fresh serum or in serum containing Mg^{2+}-EGTA fixed equivalent amounts of C3 (Fig. 2). As expected, bacteria incubated in serum containing EDTA exhibited background levels of C3 fixation. These data indicate that *L. pneumophila* fixes C3 via the alternative pathway of complement activation.

C3 Is Fixed Selectively to an Acceptor Molecule on the L. pneumophila Surface. Complement component C3 is a 195-kD protein molecule composed of two disulfide-linked chains: a 120-kD α chain, and a 75-kD β chain (Fig. 3). Fixation of C3 to the bacterial surface entails the formation of a covalent bond between the active thiolester site on the C3 α chain and a C3 acceptor molecule on the bacterium. After fixation, the C3b thus formed may be further processed to $iC3b_1$ and $iC3b_2$ upon sequential cleavage of the α chain. These C3-derived fragments remain covalently attached to the surface acceptor molecule and contain ever smaller portions of the C3 α chain. Under specific conditions not present in our experiments, $iC3b_2$ may be further degraded.

To explore C3 fixation to the *L. pneumophila* surface further, we used immunoblot analysis to search for covalent complexes of *L. pneumophila* acceptor molecules and C3. We incubated *L. pneumophila* with serum, vigorously washed the bacteria, separated the bacterial proteins by SDS-PAGE, blotted them onto nitrocellulose paper, and probed the blots for C3-containing molecules. We reasoned that under reducing conditions, both the disulfide bond linking the α and β chains of C3 and the disulfide bond linking the 43- and 46-kD α chain fragments to the portion of the α chain covalently bound to an acceptor molecule would be broken (Fig. 3). This would liberate the 75-kD β chain, and if iC3b is formed, the 46-kD ($iC3b_1$) and 43-kD ($iC3b_2$) α chain fragments (from the α chain–acceptor molecule complex). If C3b is formed, the α chain–acceptor molecule complex would have a molecular mass equal to the molecular mass of the acceptor molecule plus 110 kD. If iC3b is formed, the complex would have a molecular mass of the acceptor molecule plus 68 kD.

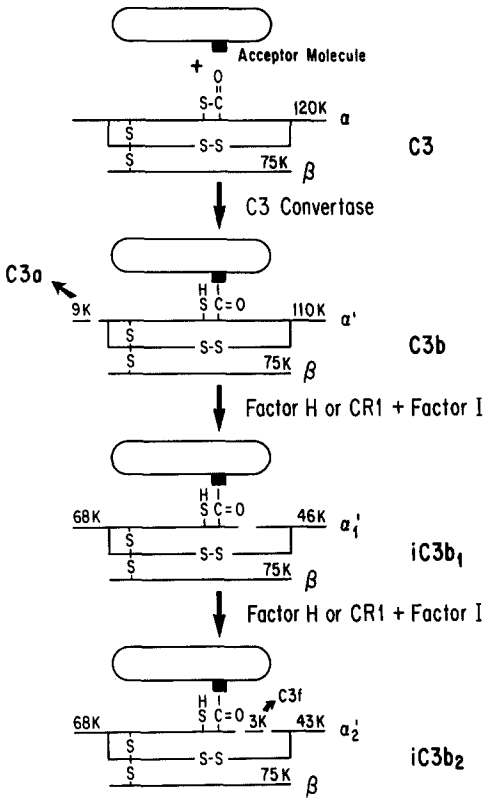

Figure 3. Fixation and cleavage of complement component C3 to a putative acceptor molecule. C3 consists of a 120-kD α chain and a 75-kD β chain linked by a disulfide bridge. C3 convertase, formed as a result of classical or alternative complement pathway activation, cleaves C3 to C3b, releasing C3a. The cleavage activates an internal thiolester within the α chain of C3b, and this thiolester forms covalent bonds with available COOH or NH_2 groups of appropriate acceptor molecules. Once fixed to an acceptor molecule, the α chain of C3b may undergo additional processing. In the presence of serum factor H or of CR1, factor I cleaves the α chain of C3b generating $iC3b_1$. Under the same conditions, factor I cleaves the α chain at an additional site near the site of the first cleavage, generating $iC3b_2$. This liberates C3f, a 3-kD fragment, from the 46-kD portion of the α chain, reducing it to 43 kD.

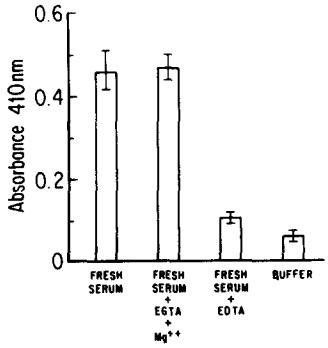

Figure 2. *L. pneumophila* fixes C3 by the alternative pathway of complement activation. *L. pneumophila* was incubated for 60 min at 37°C in fresh nonimmune human serum, in PBS, in fresh serum containing 10 mM EDTA, or in fresh serum containing 10 mM EGTA and 7 mM $MgCl_2$. The washed whole bacteria were used as antigen in an ELISA for C3, as in Fig. 1. Data are the mean ± SD for triplicate measurements.

Immunoblot analysis confirmed that C3 was fixed to the bacterium, and revealed that C3 was fixed selectively to a specific acceptor molecule (Fig. 4). Immunoblots probed with polyclonal anti-C3 antibodies revealed the presence of the 75-kD β chain, the 43- and 46-kD fragments of the α chain of iC3b, and a prominent new 150-kD C3-containing molecule, presumably consisting of the C3 α chain covalently linked to an *L. pneumophila* acceptor molecule (Fig. 4, lane *A*). The C3-containing bands formed during opsonization of viable, freshly thawed bacteria were identical in both molecular mass and amount to those formed during opsonization of bacteria passed one time on agar and freshly harvested. Immunoblots probed with an anti-iC3b mAb that recognizes a site on the

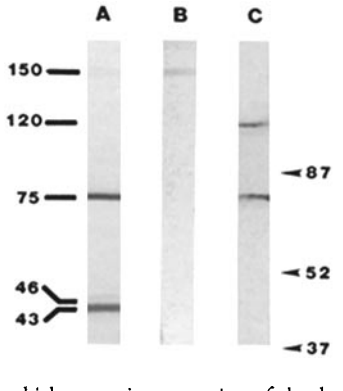

Figure 4. Opsonized *L. pneumophila* contain a high molecular weight C3-acceptor molecule complex. *L. pneumophila* was opsonized in 5% fresh nonimmune serum for 15 min at 37°C, and PMSF was added to prevent proteolytic processing of fixed complement. The bacteria were washed, subjected to SDS-PAGE on a 7.5% polyacrylamide–4 M urea gel, and blotted to nitrocellulose. The blots were probed with polyclonal anti-C3 (lane *A*) or an anti-iC3b mAb (lane *B*), which recognizes a portion of the denatured α chain near the thiolester linkage site. Lane *C* contains a native C3 standard (α chain, 120 kD; β chain, 75 kD) probed with polyclonal anti-C3 antibody. Molecular mass standards in kilodaltons are indicated next to the figure.

α chain of iC3b (and denatured C3b) also revealed the presence of the 150-kD band, confirming that this band contains the relevant portion of the C3 α chain (Fig. 4, lane *B*). Whether the 150-kD band contains the 110-kD α chain of C3b or the 68-kD α chain of iC3b linked to an acceptor molecule could not be determined by this assay.

To examine the possibility that polyclonal anti-C3 antibodies crossreact with *L. pneumophila* molecules, we probed immunoblots of unopsonized *L. pneumophila* with anti-C3. Neither the polyclonal goat anti-C3 antibody nor a more sensitive polyclonal chicken anti-C3 antibody (see below) detected bands on these immunoblots, ruling out the possibility of a crossreaction between C3 and *L. pneumophila*.

To further confirm these results, we added radiolabeled C3 to serum during the opsonization step and obtained autoradiographs of the electrophoresed proteins. The autoradiographs revealed the presence of the 150-kD band (data not shown). The autoradiograph also revealed the presence of a 110-kD band that we subsequently observed on immunoblots of opsonized *L. pneumophila* and MOMP-liposomes when we used a more sensitive chicken anti-C3 antibody (see Fig. 7 below).

The thiol ester site in the C3 α chain can undergo two separate reactions: one is the formation of an ester linkage with oxygen-containing moieties, the other is the formation of an amide bond with nitrogen-containing species. The O-linked ester bond is labile to disruption by strong nucleophiles (such as hydroxylamine or methylamine). The amide linkage is not susceptible to such attack (16, 17). This affords a convenient way to determine the type of linkage formed in the C3-acceptor molecule complex. To explore this, we opsonized *L. pneumophila* for 15 min at 37°C in fresh serum, washed the bacteria vigorously, incubated them in 1.0 M hydroxylamine in 1% SDS for 30 min at 37°C, washed them, and immunoblotted as described above. Samples of the hydroxylamine-treated bacteria contained all the C3 bands seen in the untreated samples, with no significant decrease in amount (data not shown). This same treatment released C3 from the surface of opsonized group B *Streptococcus*. This indicated that C3 fixation to the *L. pneumophila* acceptor molecule takes place through an amide bond and suggested that the acceptor molecule is a protein rather than a carbohydrate.

MOMP on Nitrocellulose Blots Uniquely Fixes C3. To explore which *L. pneumophila* component serves as an acceptor molecule for C3, we blotted samples of whole *L. pneumophila*, *L. pneumophila* membranes, isolated MOMP, and isolated LPS, then incubated the blot itself in fresh serum, washed it, and probed the blot for C3 with anti-C3 antibody (Fig. 5 *A*). C3 bound exclusively to MOMP on the blot (Fig. 5 *A*, lanes *a–d*). C3 bound to both the monomeric 29-kD MOMP band in purified MOMP (Fig. 5 *A*, lane *b*), whole membranes (lane *c*), and whole bacteria (lane *d*), as well as to a higher order MOMP aggregate found only in whole bacteria (lane *d*, *arrowhead*). C3 even bound to the small amount of MOMP that routinely contaminates the LPS preparation (lane *a*). The anti-C3 antibody does not detect MOMP or any other molecule in blotted samples of unopsonized *L. pneumophila* (data not shown).

The identification of the 29-kD C3-fixing band as MOMP was confirmed by probing a duplicate blot (untreated with serum) with an anti-MOMP antibody that recognizes only monomeric MOMP (Fig. 5 *B*). Another anti-MOMP antiserum, raised against a reduced, denatured form of MOMP, does recognize the higher order aggregates of MOMP in whole bacteria (data not shown). In contrast to MOMP, the LPS of *L. pneumophila* (the other major molecule on the surface of the bacteria) does not fix C3. The location of LPS was revealed on a third blot probed with anti-LPS antibody (Fig. 5 *C*). The LPS ladder can be clearly seen as a series of closely spaced bands rising from just behind the dye front of the blot. The entire LPS ladder (with its contaminating MOMP) displays a distinctly different pattern than blots stained with anti-C3 (Fig. 5 *A*) or anti-MOMP antibody (Fig. 5 *B*). These data show that MOMP selectively fixes C3.

The finding that a protein and not LPS fixes C3 is consistent with the results of the hydroxylamine release study, which indicated that C3 is covalently bound to a protein.

MOMP-containing Liposomes Fix C3. Our finding that MOMP on immunoblots selectively fixes C3 prompted us to explore the capacity of liposomes with isolated MOMP incorporated into their membranes to fix C3 and serve as a model system for study of *L. pneumophila* phagocytosis. We reconstituted purified MOMP into unilamellar liposomes constructed of PC and PE at a 4:1 ratio (wt/wt). This particular lipid mixture is considered to be in the fluid state and was used in the studies that demonstrated that MOMP forms a functional porin in artificial membranes (9). We confirmed by an ELISA that MOMP was present in liposomes reconstituted with MOMP (Fig. 6 *B*).

We then used the ELISA for C3 to determine the capacity of MOMP-containing liposomes fo fix C3. We incubated liposomes containing MOMP and liposomes not containing MOMP (plain liposomes) in fresh serum in the presence or absence of EDTA, washed them twice in buffer, and probed for C3 by ELISA. All liposome preparations contained equivalent amounts of total phospholipid. As seen in Fig. 6 *A*, MOMP-containing liposomes fixed almost 10 times as much C3 as plain liposomes lacking MOMP. C3 fixation by MOMP-

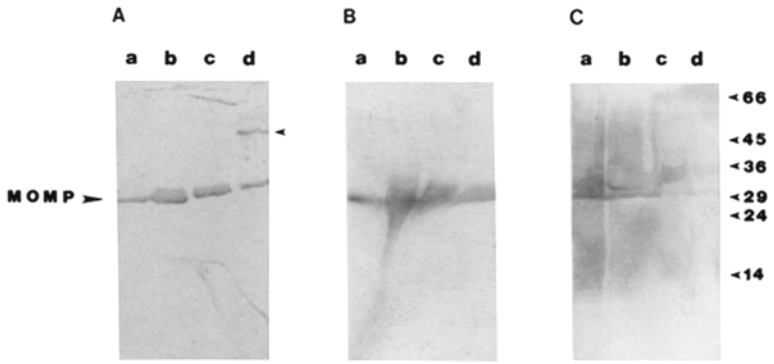

Figure 5. C3 fixes selectively to MOMP in blotted samples of whole bacteria, whole membranes, purified MOMP, and purified LPS. Whole bacteria (lanes *d*), whole *L. pneumophila* membranes (lanes *c*), purified MOMP (lanes *b*), and purified LPS (lanes *a*) were subjected to SDS-PAGE on 14% polyacrylamide–4 M urea gels and blotted to nitrocellulose. (*A*) The blot was incubated in 10% fresh serum for 60 min at 37°C, washed vigorously, and probed with polyclonal anti-C3 antibody. (*B* and *C*) The blots were not treated with serum. *B* was probed with anti-MOMP antibody, and *C* was probed with anti-LPS antibody. Molecular mass standards in kilodaltons are indicated to the right of *C*.

liposomes was abolished in serum containing EDTA, as expected. Interestingly, the small amount of C3 fixed by plain liposomes also decreased in serum containing EDTA, suggesting that this fixation results from low-level activation of C3, and does not represent nonspecific sticking of C3 to liposomes.

MOMP-Liposomes Form a C3 Complex of the Same Apparent Molecular Weight as that Formed on Intact L. pneumophila. To evaluate C3 acceptor molecule complexes formed on MOMP-liposomes, we performed immunoblotting experiments comparing opsonized MOMP-liposomes, opsonized plain liposomes, and opsonized *L. pneumophila*. We incubated *L. pneumophila* and liposomes in fresh serum with and without EDTA, washed the particles, blotted the proteins, and probed the blots for C3 (Fig. 7). In these studies, we used a polyclonal chicken anti-C3 antibody instead of a polyclonal goat anti-C3 antibody used in the earlier studies because it appeared to have greater sensitivity. With whole opsonized *L. pneumophila* (Fig. 7, lane *A*), the chicken anti-C3 antibody detected not only the high molecular mass 150-kD band seen in Fig. 4, but also a lower molecular mass band of ~110 kD. It also detected the 75-kD β chain and 43- and 46-kD α chain fragments. With MOMP-liposomes (Fig. 7, lane *B*), the chicken anti-C3 antibody detected a prominent 150-kD band of the same apparent molecular weight as the one detected on whole opsonized *L. pneumophila*; in addition, this antibody detected the 75- and 43-kD bands. With plain liposomes (Fig. 7, lane *C*), the chicken anti-C3 antibody detected only faint 75- and 43-kD bands; the 150-kD band was absent. The presence of a small amount of the 43-kD α chain fragment of C3 on plain liposomes confirms that plain liposomes fix some C3 specifically, as suggested by the ELISA data shown in Fig. 6. The absence of the 150-kD band on plain liposomes confirms that this band on MOMP-liposomes is MOMP dependent. On duplicate blots, the anti-iC3b mAb that recognizes only the portion of the denatured α chain attached to an acceptor molecule also detected the 150-kD band on *L. pneumophila* and MOMP-liposomes, confirming that this band contains the α chain of C3. The addition of EDTA to the serum abolished C3 fixation to intact *L. pneu-*

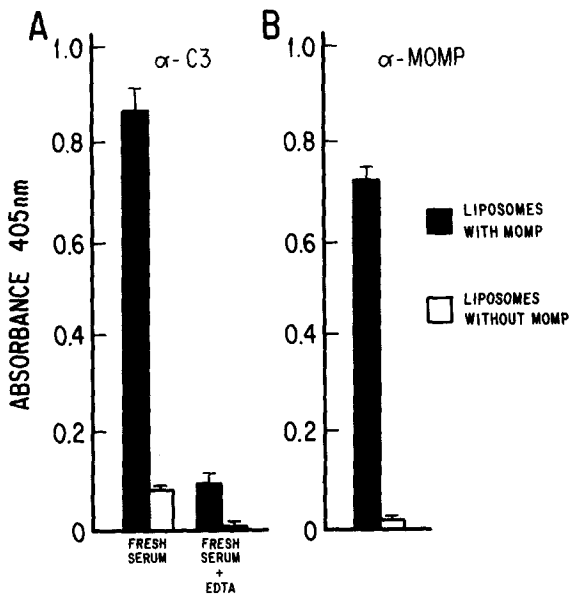

Figure 6. MOMP-containing liposomes fix C3. (*A*) Liposomes with incorporated MOMP (*solid bars*) or liposomes without MOMP (*open bars*) were incubated in fresh nonimmune human serum with and without 10 mM EDTA for 60 min at 37°C. The liposomes were harvested by ultracentrifugation, washed to remove unbound serum components, and used as antigen in an ELISA for C3. (*B*) Liposomes with MOMP (*solid bars*) or without MOMP (*open bars*) were used as antigen in an ELISA for MOMP.

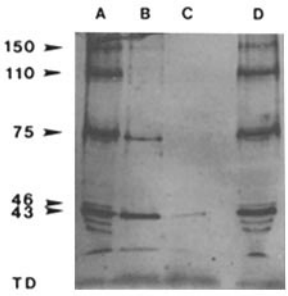

Figure 7. Opsonized *L. pneumophila* and opsonized MOMP-liposomes contain C3-acceptor molecule complexes of similar molecular weight. Whole *L. pneumophila* (lanes *A* and *D*), MOMP-liposomes (lane *B*), or plain liposomes (lane *C*) were opsonized in 10% fresh serum at 37°C for 60 min, harvested, washed twice in PBS, subjected to SDS-PAGE on a 7.5% polyacrylamide–4 M urea gel, and blotted onto nitrocellulose. The blot was probed with a polyclonal chicken anti-human C3 antibody. Molecular mass standards are indicated in kilodaltons. TD, tracking dye.

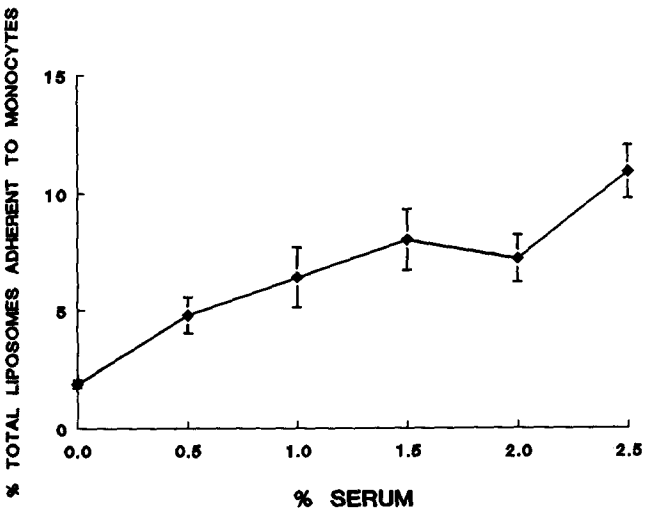

Figure 8. Opsonized MOMP-liposomes adhere to human monocytes. ^{125}I-MOMP-liposomes were opsonized for 60 min at 37°C in various concentrations of serum. The liposomes were thoroughly washed, and then added to freshly isolated adherent monocytes as described in the text. After 60 min, the coverslips were thoroughly washed, and monocyte-associated radioactivity was counted. All experiments were performed in triplicate. The percentage of counts retained on the coverslips are plotted as a function of the serum concentration used to opsonize the liposomes. Nonspecific binding of opsonized liposomes to coverslips in control wells not containing monocytes ranged from 1.2 to 1.5%. Bars represent the SEM.

mophila and to both MOMP-liposomes and plain liposomes (data not shown).

Opsonized MOMP-Liposomes Adhere to Human Monocytes. The finding that MOMP-liposomes fix C3 and form C3-MOMP complexes that are similar or identical to those formed on intact *L. pneumophila* prompted us to examine if MOMP and C3 by themselves could mediate adherence to and phagocytosis of liposomes by human monocytes. To assay adherence, we prepared MOMP-liposomes with radioiodinated MOMP, opsonized them in various concentrations of serum, washed them, incubated them with monocytes, and measured monocyte-associated radioactivity (Fig. 8). Adherence of MOMP-liposomes to monocyte monolayers was serum dependent, increasing from a level of ~2% of added liposomes in the absence of serum to a level of almost 11% in the presence of 2.5% serum in the representative experiment shown in Fig. 8. Adherence of opsonized liposomes to coverslips not containing monocytes in control wells was independent of the serum concentration used for opsonization, and ranged from 1.2 to 1.5%. These data demonstrate that MOMP and C3 can mediate monocyte adherence of liposomes and that MOMP-liposomes can serve as a model target particle for studies of C3-dependent adherence by monocytes.

Opsonized MOMP-Liposomes Are Phagocytized by Human Monocytes. To determine if monocytes phagocytize opsonized MOMP-liposomes, we examined the interaction of mono-

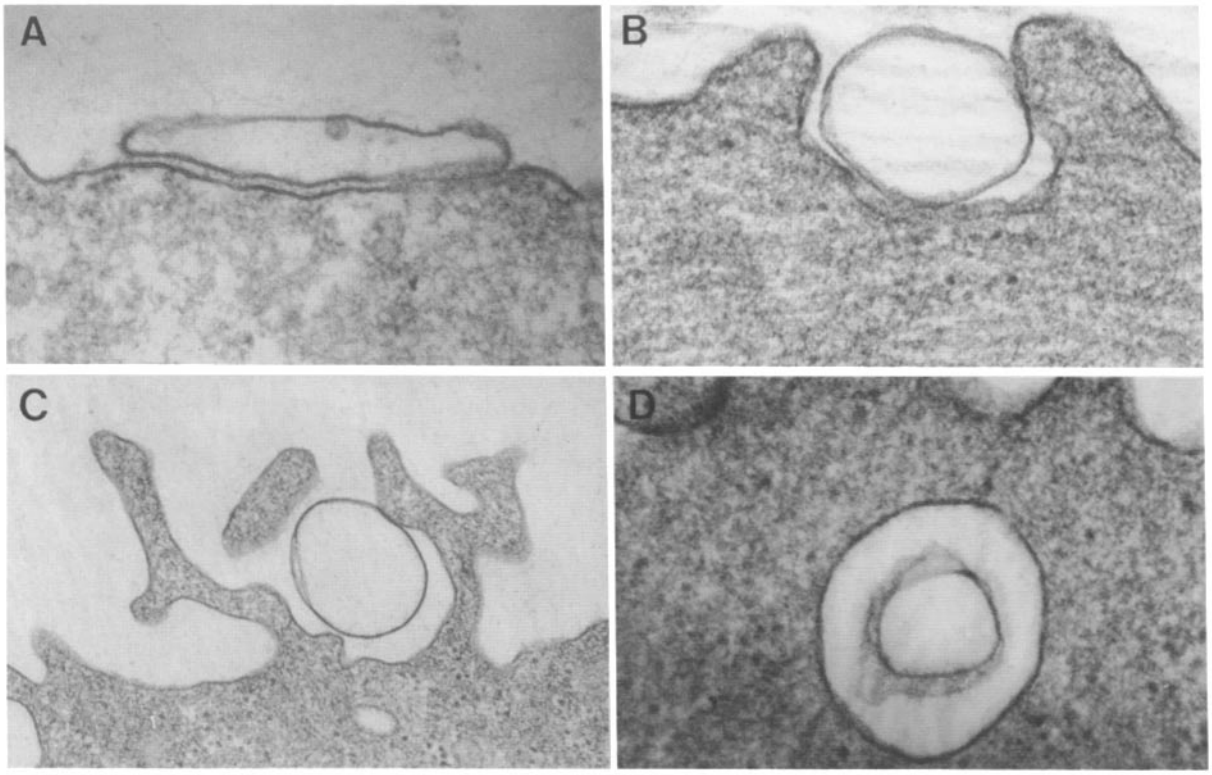

Figure 9. Opsonized MOMP-Liposomes are phagocytized by human monocytes. MOMP-liposomes were opsonized for 60 min at 37°C in 10% serum, thoroughly washed, incubated with freshly isolated mononuclear cells, and processed for electron microscopy as described in the text. (*A–D*) Sequential stages in monocyte phagocytosis of MOMP-liposomes. (*A*) At an early stage in the ingestion process, the MOMP-liposome is tightly adherent to the monocyte surface. (*B* and *C*). At later stages of ingestion, MOMP-liposomes are in the process of being internalized by monocyte pseudopods extending circumpherentially about them. (*D*) In the final stage of ingestion, the MOMP-liposome is completely enclosed in a membrane-bound vacuole. Magnification: *A*, ×100,000; *B*, ×76,000; *C*, ×48,000; and *D*, ×96,000.

cytes and opsonized MOMP-liposomes by electron microscopy. MOMP-liposomes, opsonized and washed, were layered over a pellet of mononuclear cells and centrifuged on top of them at 0°C. The samples were shifted to 37°C, incubated for 3–6 min, and processed for electron microscopy (Fig. 9). Opsonized MOMP-liposomes were avidly ingested by monocytes. In contrast, although accurate quantitation was not possible in this assay, opsonized plain liposomes were infrequently ingested by monocytes, consistent with the much lower level of C3 fixation of these liposomes. MOMP-liposomes incubated in serum containing EDTA were rarely if ever ingested by monocytes.

Most of the MOMP-liposomes were located intracellularly in monocytes. However, many were observed in the process of being internalized (Fig. 9). These liposomes were ingested by a conventional form of phagocytosis, i.e., monocyte pseudopods were observed fixed in the process of moving circumferentially and more or less symmetrically about the liposomes.

Discussion

Previous studies have demonstrated that complement receptors CR1 and CR3 mediate phagocytosis of *L. pneumophila* and have strongly indicated that fragments of complement component C3 on the bacterium serve as ligands for these receptors. However, evidence that C3 in nonimmune serum fixes to *L. pneumophila* has been lacking. The current study confirms that the bacterium does indeed fix C3 under these conditions. The whole cell ELISA, which used opsonized *L. pneumophila* as antigen, demonstrated that C3 is fixed to the bacterium and that fixation takes place by the alternative pathway of complement activation. Immunoblot analysis confirmed that C3 is fixed to the *L. pneumophila* surface and processed under our experimental conditions to at least the iC3b stage.

Previous immunofluorescence studies did not demonstrate complement fixation by *L. pneumophila* in nonimmune serum (8). In the present study, we suspect that the greater sensitivity of the ELISA allowed us to detect a very small quantity of fixed C3. Consistent with this idea, comparative assays of opsonized *L. pneumophila*, opsonized *Escherichia coli* 09:K29:H- (18), and opsonized zymosan have revealed that *L. pneumophila* fixes only about one-tenth the amount of C3 as does *E. coli* or zymosan (Bellinger-Kawahara and Horwitz, unpublished data).

Our study also demonstrates that C3 fixes selectively to MOMP on the surface of *L. pneumophila*. Immunoblot analysis to opsonized *L. pneumophila* revealed a unique major C3-acceptor molecule complex of ~150 kD and, with the more sensitive chicken anti-C3 antibody, a second potential complex of ~110 kD. These same two bands can be seen in autoradiograms of intact *L. pneumophila* opsonized in fresh serum spiked with radioiodinated C3. The C3-acceptor molecule complexes remained stable to hydroxylamine treatment, indicating that C3 fixation takes place via an amide rather than ester linkage. This in turn suggested that a protein rather than a carbohydrate-containing molecule such as LPS was serving as the acceptor molecule. Consistent with this, blotted samples of *L. pneumophila* and its components selectively fixed C3 to the MOMP of *L. pneumophila*. *L. pneumophila* LPS did not fix C3.

Interestingly, C3 fixation may be targeted to specific portions of the MOMP molecule. When immunoblots of cyanogen bromide fragments of MOMP are incubated in serum, some but not all MOMP fragments fix C3 (Bellinger-Kawahara and Horwitz, unpublished data).

We attempted by three independent approaches to identify MOMP directly in the C3-acceptor molecule complex formed on the surface of opsonized *L. pneumophila*. None of these attempts was wholly successful. First, we attempted on immunoblots of opsonized *L. pneumophila* to detect MOMP in the 150- or 110-kD C3-acceptor molecule complex with anti-MOMP antibody. This was unsuccessful, perhaps because the epitope recognized by the antibody was near the thiolester linkage site or otherwise masked by the large C3 α chain covalently bound to the much smaller acceptor molecule. Second, we attempted to immunoprecipitate C3-acceptor molecule complexes from solubilized preparations of opsonized *L. pneumophila* with anti-C3 or anti-MOMP antibody. These attempts were unsuccessful, presumably because of the small amount of complex formed. Third, we attempted to identify the acceptor molecule by raising antibody to the C3-acceptor molecule complex in the hope that some antibody would be raised against the acceptor molecule portion and that this antibody would recognize the acceptor molecule on blots of unopsonized *L. pneumophila*. We eluted the 150- and 110-kD regions of SDS-PAGE gels of opsonized *L. pneumophila* and immunized rabbits with the preparations. The antisera thus obtained recognized MOMP on immunoblots. However, since MOMP forms a multimeric porin in the *L. pneumophila* outer membrane, we could not rule out the possibility that the 150- or 110-kD regions of the SDS-PAGE gels of opsonized *L. pneumophila*, which were used as antigen, were "contaminated" with a small amount of aggregated MOMP and that antibody was raised against these molecules rather than the C3-acceptor molecule complex.

Immunoblot analysis of C3-acceptor molecule complexes formed on MOMP-liposomes provided indirect evidence that the 150- and 110-kD complexes on blots of opsonized *L. pneumophila* contained MOMP. This analysis revealed that MOMP-liposomes form C3-acceptor molecule complexes in nonimmune serum of the same molecular weight (150 and 110 kD) as those formed by intact *L. pneumophila*. Liposomes lacking MOMP do not form these complexes in serum; nor do MOMP-liposomes in serum containing EDTA. This, together with our finding that only the MOMP fixes C3 on immunoblots of whole *L. pneumophila*, provides strong evidence that MOMP is not only a major molecule of *L. pneumophila* that fixes C3, but the only major C3-fixing molecule.

A C3-acceptor molecule complex consisting of C3b linked to monomeric MOMP would have a combined apparent molecular mass under reducing conditions of 139 kD (110-kD C3b α chain plus 29-kD MOMP). This combined apparent molecular mass closely approximates the apparent molecular mass of the major C3-acceptor molecule complex formed on immunoblots of opsonized *L. pneumophila* and MOMP-

liposomes (150 kD). A C3-acceptor molecule complex consisting of iC3b linked to monomeric MOMP would have a combined apparent molecular mass under reducing conditions of 97 kD (68-kD α prime chain plus 29-kD MOMP). Again, this combined apparent molecular mass closely approximates the apparent molecular mass of the minor C3-acceptor molecule complex formed on immunoblots of opsonized *L. pneumophila* and MOMP-liposomes (110 kD). The SDS-PAGE system used in these experiments is accurate within ~10% of a given molecular mass, and therefore, the small differences between the predicted and observed molecular masses of the C3-acceptor molecule complexes are within the range of experimental error.

To further explore the capacity of MOMP to fix C3 and mediate endocytosis, we reconstituted MOMP into unilamellar liposomes and used the MOMP-liposomes as a model system for opsonization and phagocytosis experiments. ELISA and immunoblot studies demonstrated that MOMP-liposomes fix a relatively large amount of C3, whereas liposomes lacking MOMP fix only a small amount of C3. Our finding that plain liposomes fix C3 is consistent with the recent report by Cunningham et al. (19), who reported that liposomes with a net positive charge and "fluid" membrane state may fix complement by the alternative pathway. For our liposomes, we selected the lipid composition that was used in earlier studies, which demonstrated that MOMP reconstituted into black lipid membranes acts as a porin and retains normal porin function (10). This composition is such that the liposomes would carry a net positive charge and have a fluid membrane state.

Liposome-MOMP complexes not only fix C3 in nonimmune serum, but once so opsonized, they adhere to and are ingested by human monocytes. The data strongly indicate that C3 mediates ingestion of MOMP-liposomes by complement receptors on the monocytes. While it is theoretically possible that other monocyte receptors are involved in the actual internalization event, the fact that MOMP and C3 are the only evident molecules present on the liposome surface suggests that complement receptors may by themselves mediate ingestion of MOMP-liposomes. If so, then complement receptors may also by themselves mediate ingestion of opsonized *L. pneumophila* and other intracellular pathogens that fix C3 in nonimmune serum, including *Mycobacterium leprae* (20), *M. tuberculosis* (21), *Leishmania donovani* (22), and *L. major* (23, 24).

Although *L. pneumophila* are ingested by coiling phagocytosis, MOMP-liposomes are ingested by conventional phagocytosis. This indicates that liposome-MOMP-C3 complexes do not meet all the requirements for inducing the coiling phenomenon.

In addition to MOMP on *L. pneumophila*, C3 acceptor molecules have been identified on two other intracellular pathogens: a 72-kD glycoprotein (gp 72) on noninfective *Trypanosoma cruzi* epimastigotes (25), and lipophosphoglycan on *L. major* (24). While these three acceptor molecules form a heterogeneous group, it may be significant that all of them are major constituents on the parasite surface.

Knowledge of the roles of complement receptors and C3 in mediating phagocytosis of *L. pneumophila* and other intracellular parasites, and the identification of C3-acceptor molecules on the surface of intracellular parasites, not only enhances our understanding of phagocytosis of these organisms, but also offers the potential of developing new strategies for preventing or treating diseases caused by these pathogens. For example, it may be possible to design immunological or other reagents that interfere with C3 fixation to its acceptor molecule or block the recognition of fixed C3 by complement receptors.

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