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Role of Complement Component C1q in Phagocytosis of *Listeria monocytogenes* by Murine Macrophage-Like Cell Lines

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***Listeria monocytogenes* is a facultative intracellular pathogen of a great variety of cells. Among them, macrophages constitute the major effector cells of listerial immunity during the course of an infection. Although the molecular bases of *L. monocytogenes* attachment and entry to phagocytes are not completely understood, it has been demonstrated that C3b significantly increases *L. monocytogenes* uptake by macrophages via complement receptor type 3. The first component of complement, C1q, is present in organic fluids at a relatively high concentration, and C1q receptor sites in macrophages are also abundant. In the present report, results of studies on the role of C1q in the internalization and infectivity of *L. monocytogenes* by macrophages are presented. *L. monocytogenes* uptake is enhanced by prior treatment of bacteria with normal sera. Heated serum or C1q-deficient serum abrogates this enhancement. Purified C1q specifically restored uptake. This effect was blocked by the addition of $F(ab')_2$ anti-C1q antibody but not by an irrelevant matched antibody. Direct binding of C1q to *L. monocytogenes* was specific, saturable, and dose dependent with both fluorescent and radiolabeled C1q. *N*-Acetyl-D-alanyl-L-isoglutamine, diaminopimelic acid, and L-rhamnose caused a significant dose-dependent inhibition of C1q binding to bacteria, suggesting that these molecules, at least, are involved in the attachment of C1q to *L. monocytogenes* cell wall. When C1q binding structures on macrophagelike cells were blocked with saturating concentrations of C1q, the uptake of C1q-opsonized bacteria was less than in untreated cells. These experiments demonstrate that, in addition to other reported mechanisms, *L. monocytogenes* binds C1q, which mediates enhanced uptake by macrophages through C1q binding structures.**

There are several molecular mechanisms by which intracellular pathogens interact with host cells. In some cases, phagocytosis takes place by direct and specific recognition between molecules on the microorganism surface and complementary structures on the phagocyte surface (3, 17, 29, 42). However, in most cases, specific molecules act as bridges between the surface of the microorganism and specific receptors on the phagocyte surface. C3 and complement receptors may represent one of the most important receptor-mediated mechanisms in the recognition of parasites by phagocytes. Among such intracellular pathogens are *Leishmania* species, *Histoplasma capsulatum* (6), *Legionella pneumophila* (30), *Mycobacterium tuberculosis* (37), and *Mycobacterium leprae* (38), each of which can be phagocytosed via CR1, CR3, and/or CR4. Less well studied is the role of C1q receptor in the entry of pathogens into the host cells. A role for C1q in the phagocytosis of pathogens, probably through the C1q receptor, has been suggested for *Trypanosoma cruzi* (34), *Schistosoma mansoni* (36), *Salmonella minnesota* (35), and *Treponema pallidum* (4).

Listeria monocytogenes, a gram-positive facultative intracellular bacterium, can infect a great variety of cells, such as epithelial cells, fibroblasts, hepatocytes, and cells from the mononuclear phagocyte system. In experimental infections, mononuclear phagocytes constitute the major effector cells of immunity against *Listeria* spp. The reason for the rapid and selective uptake of *L. monocytogenes* by macrophages (Mϕ), however, has not yet been resolved. The existence on mammalian cells of specific receptors for molecules expressed on the cell wall of *L. monocytogenes* has been speculated. Recently, it has been reported that *L. monocytogenes* is opsonized by C3 and that its uptake is mediated by CR3 (9).

C1q is present in serum and other body fluids in significantly high concentrations, and because of its abilities to interact with extracellular matrix proteins such as laminin, fibronectin, and collagen, it significantly enhances ingestion of microorganisms (4, 41). On the other hand, C1q is a component not well studied in gram-positive bacteria. This is probably because gram-positive bacteria do not activate the classical pathway of complement directly and complement does not play a role in the direct killing of these bacteria (18). For these reasons, we have studied the role of C1q in the uptake of *L. monocytogenes* by Mϕ-like cell lines. We report here that (i) specific structures of *L. monocytogenes* bind C1q, (ii) phagocytosis of C1q-opsonized *L. monocytogenes* is significantly enhanced, and (iii) this effect seems to be mediated by specific C1q receptors on Mϕ.

(These findings were presented previously in preliminary form [1]).

MATERIALS AND METHODS

Bacteria. *L. monocytogenes* LO28 serovar 1/2c used in this study has been described elsewhere (47) and was kindly provided by J. C. Pérez-Díaz (Hospital Ramon y Cajal, Madrid, Spain). The *Listeria* strain was grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C with aeration. The bacteria were obtained in the logarithmic

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phase of growth and were stored at −70°C in phosphate-buffered saline (PBS) with 20% (vol/vol) glycerol until used.

Cell lines. The Mϕ-like cell lines IC-21 and P-388D1 were obtained from the American Type Culture Collection. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and gentamicin (50 μg/ml) (R10 medium). All media and buffers were confirmed to be endotoxin-free (<0.01 ng/ml) by a chromogenic *Limulus* amebocyte lysate microassay from Whittaker M. A. Bioproducts (Walkersville, Md.).

Sera. Normal human serum (NHS) was collected and frozen in aliquots at −70°C. Some samples of serum were heated at 56°C for 30 min to inactivate complement. Human C1q-deficient serum (C1qDS) was obtained from patients with a complete C1q deficiency, described previously (24).

Purification of C1q complement component. C1q was isolated from human donor plasma as reported previously (24). All C1q preparations were homogeneous as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% gels under reduced conditions and stained with Coomassie brilliant blue.

Preparation of an antibody against C1q. An antiserum raised in rabbits against C1q was obtained commercially (Sigma Chemical Co., St. Louis, Mo.). The immunoglobulin G (IgG) fraction was purified by standard procedures, and then $F(ab')_2$ fragments of this antibody were prepared by digestion of the purified IgG with pepsin at pH 4.5 (18 h, 37°C) (16). $F(ab')_2$ fragments were separated from undigested IgG by using a protein A-Sepharose column (Pharmacia). For control purposes, rabbit IgG $F(ab')_2$ of irrelevant specificity was used.

Fluorescence labeling of C1q. C1q was labeled with fluorescein isothiocyanate (FITC) (Sigma) by a standard dialysis technique for preparing fluorescent antibodies. Briefly, a 1-ml solution of C1q (1 mg/ml) was dialyzed against a 10-ml solution of FITC (0.1 mg/ml) in PBS for 18 h at 4°C with constant stirring. The conjugated C1q solution (FITC-C1q) was dialyzed against PBS until fluorescein was no longer detectable in the dialysate and then filtered through Sephadex G-25 to separate FITC-C1q from free FITC.

Radiolabeling of C1q. Radiolabeling was done by the method of Bolton and Hunter (specific activity of ^{125}I, 185 Bq/ml; Amersham). Purified human C1q (1 mg/ml) was washed with PBS by gel filtration. Then, 300 μg of C1q was added to the dried iodinated ester and allowed to react (2 h, 4°C) in phosphate buffer. ^{125}I-C1q was separated from the nonconjugated iodinated ester by filtration through a Sephadex G-50 column. Typical preparations were labeled to a specific activity of 3.22 MBq/μg.

Uptake of *L. monocytogenes*. The ability of the Mϕ-like cell lines to take up *L. monocytogenes* was measured by a previously described assay (20) with some modifications. Briefly, Mϕ were plated in 96-well tissue culture plates (Costar, Cambridge, Mass.) at 2×10^6 cells per ml the evening before use. An aliquot of frozen *L. monocytogenes* was thawed, and 2×10^6 bacteria per well (0.1 ml per well) were added to the culture plates. The plates were then centrifuged at $1,000 \times g$ for 7 min. After 15 min at 37°C to allow the uptake of bacteria, free bacteria were removed by several washes with cold PBS. They were then incubated (45 min, 37°C) in RPMI 1640 plus 5 μg of gentamicin per ml. (This gentamicin concentration, present in the culture for 45 min, kills all extracellular *L. monocytogenes*. The end of this incubation period was considered time zero.) Monolayers were washed three times with Hanks' balanced saline solution, and the same volume of RPMI 1640 (R0) was added. The cells were lysed by several cycles of freezing and thawing. The number (CFU) of viable bacteria per well was determined by quantitative plate counts on blood agar plates (Columbia blood agar; Becton Dickinson, San Jose, Calif.) after 24 h of incubation. Each result is given as the mean of three determinations.

In some experiments, the effect of different sera (NHS, decomplemented NHS, or C1qDS) on the *L. monocytogenes* uptake by Mϕ was evaluated. Therefore, 2×10^7 bacteria per ml were preincubated (20 min, 4°C) with 20% sera diluted in R0 and washed once by centrifugation at $12,000 \times g$ for 5 min. These pellets were then suspended in the proper amount of R0. Bacteria were centrifuged onto the cell monolayers as described previously. In other experiments, *L. monocytogenes* was preincubated as described above with C1qDS or purified C1q (100 μg) in the presence or absence of 200 μg of rabbit $F(ab')_2$ anti-human C1q.

Other experiments in which C1q receptors were saturated with purified C1q were performed. After the cell monolayers were plated in R0, they were incubated in the presence or absence of 60 μg of C1q (an amount found to be enough to saturate all C1q binding sites; see below) for 60 min. This procedure was done in parallel experiments run at both 4 and 37°C. Then, cells were infected with *L. monocytogenes* (preopsonized with C1q or not preopsonized) as described above. Excess C1q was removed from both cells and bacteria by a wash step. Control levels were defined by the values obtained with nonopsonized *L. monocytogenes* and untreated Mϕ.

Fluorescence localization of C1q deposition onto *L. monocytogenes*. Fifty micrograms of FITC-C1q was incubated (30 min, 37°C) with 2×10^7 bacteria per ml. This was followed by a centrifugation step ($12,000 \times g$, 5 min, 4°C) to eliminate the unbound FITC-C1q. Bacteria were washed twice with PBS. FITC-C1q-opsonized *L. monocytogenes* cells were added to Mϕ cells as described for the uptake assay. Cells were fixed with 70% (vol/vol) methanol, incubated with a rabbit anti-*Listeria* antiserum, and then incubated with rhodamine-labeled goat $F(ab')_2$ anti-rabbit antibody. Double staining was evaluated by colocalization of both red staining (related to *L. monocytogenes*) and green staining (related to C1q bound to *L. monocytogenes*) on the same structures. $F(ab')_2$ anti-C1q, purified C1q, and/or 10 mM EDTA incubated with FITC-C1q was used as a negative control.

Assay for C1q binding on Mϕ, using flow cytometry. IC-21 cells cultured in R10 medium were washed and resuspended in PBS–2% fetal calf serum. In a typical experiment, different sets of cells were incubated with FITC-C1q with or without unlabeled C1q for 60 min at 4°C. After washing, cells were fixed with 1% paraformaldehyde. The samples were run on a FACScan flow cytometer (Becton Dickinson) equipped with a 15-mW, 488-nm, air-cooled argon ion laser. Daily performance was monitored by using Calibrite beads (Becton Dickinson). A total of 2,000 to 9,000 events per sample was collected by four-decade log amplification. Data were analyzed on an HP9000 series model 310 computer, and histograms were generated with LYSIS software.

^{125}I-C1q binding to *L. monocytogenes*. About 6×10^7 bacteria per ml were incubated (20 min, 0°C) with various amounts of ^{125}I-C1q in a total volume of 0.7 ml. Aliquots, 100 μl, of the reaction mixtures were layered over 150 μl of an oil cushion (60% dibutyl phthalate, 40% dioctyl phthalate [Eastman Kodak Co., Rochester, N.Y.]), and the mixtures were centrifuged ($10,000 \times g$, 60 s, 4°C). The tip of the tube, containing the cell pellet, was cut with a Mozart razor blade, and the radioactivity was counted. Variable concentrations

of bacteria (6×10^4 to 6×10^7 bacteria per ml) were used to calculate the specificity of the binding assay.

Inhibition assays of C1q-*L. monocytogenes* binding. Inhibition assays of C1q-*L. monocytogenes* binding by different *L. monocytogenes* cell wall constituents were performed. In brief, 6×10^7 bacteria per ml were incubated with a fixed amount of ^{125}I-C1q (0.2 μg) and various amounts of the following cell wall components: diaminopimelic acid, L-rhamnose, D-galactose, D-mannose, muramic acid, *N*-acetyl-muramyl-L-alanyl-D-isoglutamine, *N*-acetyl-muramyl-D-alanyl-D-isoglutamine, and *N*-acetyl-muramyl-L-alanyl-L-isoglutamine. All of these reagents were from Sigma. Human collagen type I (Calbiochem, San Diego, Calif.) was employed as a potential inhibitor because of its structural analogy with C1q (33, 34). Finally, to set a positive control of the binding inhibition, various amounts of unlabeled C1q were also assayed.

^{125}I-C1q binding to cellular receptors. Both IC-21 and P-388D1 cells (10^6) were incubated with increasing amounts (0.18 to 18 μg) of ^{125}I-labeled C1q in a total volume of 100 μl. After incubation (2 h, 4°C), 80 μl of the cell suspension was applied to 200 μl of a fetal calf serum cushion, and the mixture was centrifuged (10,000 × *g*, 60 s, 4°C). For dilutions and also for washings, PBS (pH 7.5) buffer containing 2% fetal calf serum and 0.01% NaN_3 was employed. Tubes were sectioned with a Mozart razor blade, and cell pellets and supernatants were counted in a gamma counter. Specific binding was defined as the difference between the total binding and the nonspecific binding occurring in the presence of a 250-fold excess of unlabeled C1q. The assays were performed in duplicate. To calculate receptor number and binding affinity, a Scatchard analysis was performed.

Statistical analysis. Data are expressed as means ± standard deviations (SD). Differences between conditions were statistically determined by Student's *t* test.

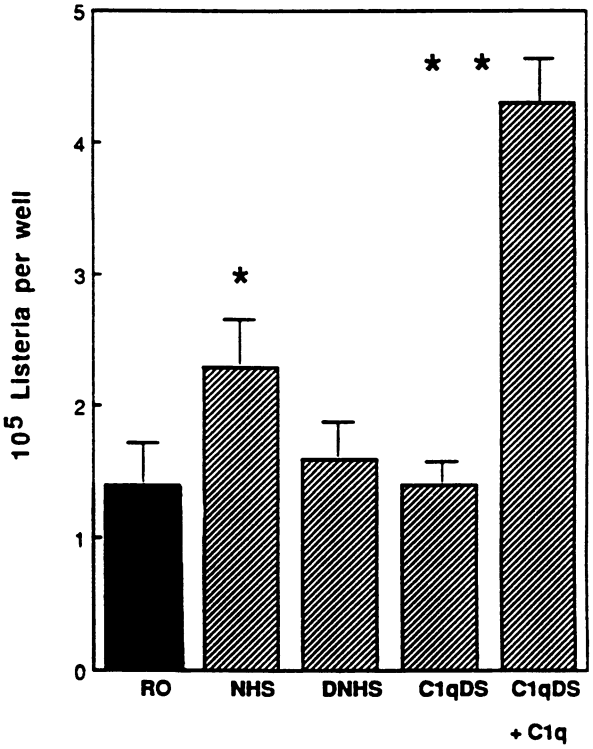

FIG. 1. Effect of serum on *L. monocytogenes* uptake by IC-21 Mϕ. Bacteria, 10^7, were preincubated with different sera before the infection step. R0, RPMI medium alone; DNHS, decomplemented NHS; C1qDS, C1q-deficient serum. Purified C1q was added at 100 μg/ml. Results are expressed as mean CFU ± SD of triplicate experiments. Asterisks indicate significant modifications compared with R0: *, $P < 0.05$; **, $P < 0.005$.

RESULTS

Effect of serum on uptake of *L. monocytogenes* by Mϕ. Initial experiments showed that the uptake of *L. monocytogenes* by Mϕ in serum-free medium (R0) was potentiated by preincubation of bacteria in NHS. Heating the serum to destroy complement or using C1qDS abrogates the increase of bacterial entry into Mϕ. When C1qDS was reconstituted with purified C1q, the serum enhancement was restored and even significantly increased (Fig. 1). These results pointed to some complement component, presumably C1q, as the responsible factor of serum enhancement in *L. monocytogenes* uptake by Mϕ. Moreover, there were only small differences, if any, between uptake in the presence of R0 and that in the presence of decomplemented NHS or C1qDS, suggesting that antibodies did not play a role.

Contribution of C1q to *L. monocytogenes* uptake. To clarify the involvement of C1q in *L. monocytogenes* entry into Mϕ, we investigated whether the incubation of bacteria in serum-free medium (R0) reconstituted with purified factors reproduced the increased effect seen in the presence of serum. These results show that purified C1q potentiates *L. monocytogenes* entry into IC-21 Mϕ, and such enhancement could be abrogated by the addition of $F(ab')_2$ fragments of rabbit IgG anti-C1q to the incubation medium. This enhancement was not abrogated by $F(ab')_2$ fragments of an irrelevant rabbit IgG (Fig. 2). This effect was also observed in P-388D1 cells (results not shown).

Deposition of C1q onto *L. monocytogenes*. Localization of C1q on the *L. monocytogenes* surface was evaluated by double immunofluorescence staining. *L. monocytogenes* cells were first opsonized with FITC-C1q and used to infect IC-21 cells. After fixation, *L. monocytogenes* was localized on Mϕ by using a specific rhodamine-labeled anti-*Listeria* antibody. Figure 3 shows double staining of both *L. monocytogenes* and C1q onto IC-21 cells. Panel A shows rhodamine labeling of *L. monocytogenes*, while panel B shows the staining pattern of FITC-C1q bound to *L. monocytogenes*. By comparison, C1q-opsonized *L. monocytogenes* represents approximately 30% of the total rhodamine-stained *L. monocytogenes*. C1q binding to *L. monocytogenes* was divalent cation dependent because no staining was observed when FITC-C1q and *L. monocytogenes* were incubated in the presence of 10 mM EDTA. Specificity was also proved by the absence of FITC staining when unlabeled C1q or anti-C1q antibodies were added to the incubation medium (data not shown).

Characteristics of ^{125}I-C1q binding to *L. monocytogenes*. To further characterize the C1q binding to *L. monocytogenes*, purified C1q was radiolabeled. The binding of C1q to *L. monocytogenes* at 4°C is concentration dependent and saturable (Fig. 4). The specificity of the ^{125}I-C1q binding to *L. monocytogenes* was determined by performing a competition experiment with increasing amounts of unlabeled C1q and a fixed amount of radioiodinated C1q. These results showed that the binding of ^{125}I-C1q to *L. monocytogenes* was inhibited by unlabeled C1q by almost 50% when ^{125}I-C1q and unlabeled C1q were both employed at the same concentration (Fig. 4, inset).

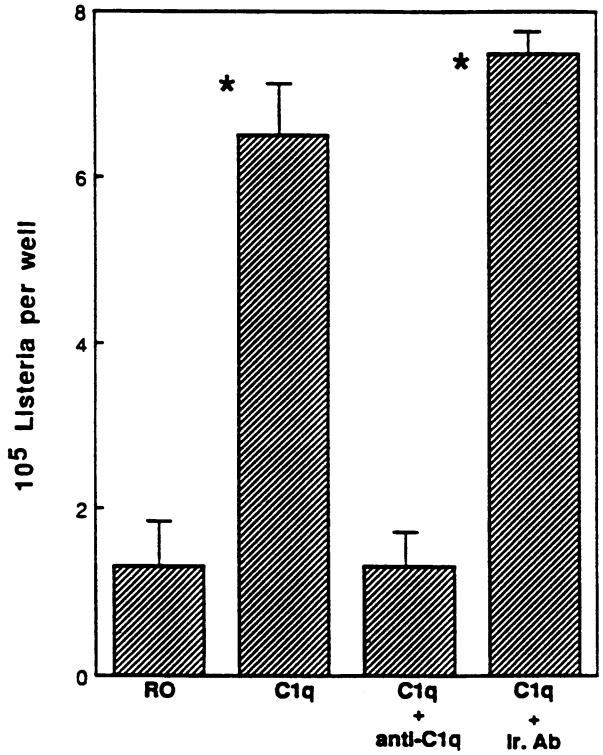

FIG. 2. Role of C1q in uptake of *L. monocytogenes* by IC-21 Mϕ. Bacteria, 10^7, were opsonized with 100 μg of purified C1q per ml in the presence or absence of an $F(ab')_2$ rabbit anti-C1q antibody (anti-C1q) or an $F(ab')_2$ rabbit IgG antibody of irrelevant specificity (Ir.Ab) before IC-21 cells were infected. Results are expressed as mean CFU ± SD of triplicate experiments. Asterisks indicate significant modifications compared with R0 ($P < 0.005$).

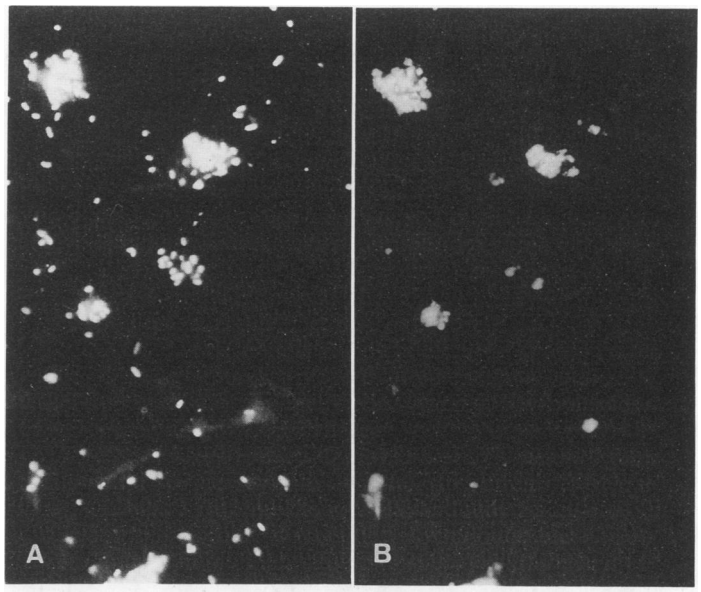

FIG. 3. Localization of C1q on *L. monocytogenes* by immunofluorescence. In this experiment, IC-21 cells were incubated with FITC-C1q-opsonized *L. monocytogenes*. After methanol fixation, coverslips were incubated with a rabbit anti-*L. monocytogenes* antiserum and then with rhodamine goat $F(ab')_2$ anti-rabbit antibody. (A) Red fluorescence due to *L. monocytogenes* staining; (B) colocalization of C1q binding on *L. monocytogenes* (green fluorescence).

Inhibition of ^{125}I-C1q binding to *L. monocytogenes* with different cell wall components. To identify the bacterial structure responsible for the C1q binding, an inhibition experiment was performed. Different concentrations of characteristic constituents of the *L. monocytogenes* cell wall (46) were used: diaminopimelic acid, muramic acid, L-rhamnose, D-galactose, D-mannose, and *N*-acetyl-muramyl-dipeptide isoforms. The highest inhibition was seen with the three major components of *L. monocytogenes* cell wall skeleton: diaminopimelic acid, L-rhamnose, and the *N*-acetyl-muramyl-L-alanyl-D-isoglutamine dipeptide. Almost no inhibition was observed with the D-D and L-L isoforms used as controls or with either D-galactose or D-mannose. No inhibition was obtained when muramic acid was used as an inhibitor. Collagen was also included in these experiments to test whether it could also bind to *L. monocytogenes* or whether the collagenlike portion of C1q (32) could be bound to the *L. monocytogenes* cell wall. Collagen does not inhibit ^{125}I-C1q binding (Table 1).

Contribution of putative C1q receptors on Mϕ to *L. monocytogenes* uptake. Having studied the binding of C1q to *L. monocytogenes* and its participation in the uptake of *L. monocytogenes* by Mϕ, further analysis of the role of C1qR in *L. monocytogenes* uptake by Mϕ was carried out. It was of interest to analyze C1q binding structures on Mϕ. In our system, ^{125}I-C1q binds to IC-21 cells in a specific and saturable manner at 4°C. More than 90% of the total binding was specific, since it was blocked in the presence of a 250-fold excess of unlabeled C1q. However, although under the applied experimental conditions (normal-ionic-strength buffer) the binding was also specific in P-388D1 cells, saturation could not be obtained (data not shown). Figure 5 shows the binding data and Scatchard plot analysis, revealing 102,000 binding sites per cell, with a binding constant of 2.09×10^7 M^{-1} in the case of IC-21 cells.

By flow cytometry, the specific binding of FITC-C1q to IC-21 cells was determined to be blocked by the addition of unlabeled C1q. In a representative experiment shown in Fig. 6, a 200-fold excess of unlabeled C1q significantly blocked FITC-C1q staining on IC-21 cells.

The C1q dependence of uptake of *L. monocytogenes* by Mϕ suggested a role for C1q binding structures on Mϕ. Although at least a putative monoclonal antibody against the C1q receptor has been described (31), no antibody has been shown to date to immunoprecipitate a specific surface component and/or to mimic the C1q-mediated effects on phagocytic cells (14).

To test this hypothesis indirectly, the ability of purified C1q to compete with C1q-opsonized *L. monocytogenes* uptake by C1q binding structures on Mϕ-like cells was examined. A representative experiment is presented in Fig. 7. These experiments were run in IC-21 and P-388D1 cells at both 4 and 37°C. It was found that the uptake of C1q-opsonized *L. monocytogenes* by IC-21 cells could be impaired by previous treatment with a blocking amount (60 μg/ml) of C1q (3×10^5 versus 7.6×10^5 CFU, respectively) (H versus F bars in Fig. 7). However, similar amounts of nonopsonized *L. monocytogenes* were taken up by both sets of IC-21 cells (4.26×10^6 CFU, C1q-treated IC-21 cells, versus 3.73×10^5 CFU, untreated cells) (E versus G bars in Fig. 7). Similar results were obtained in experiments performed with P-388D1 cells (Fig. 7, striped bars in panel at right). However, when the same experiment was carried out

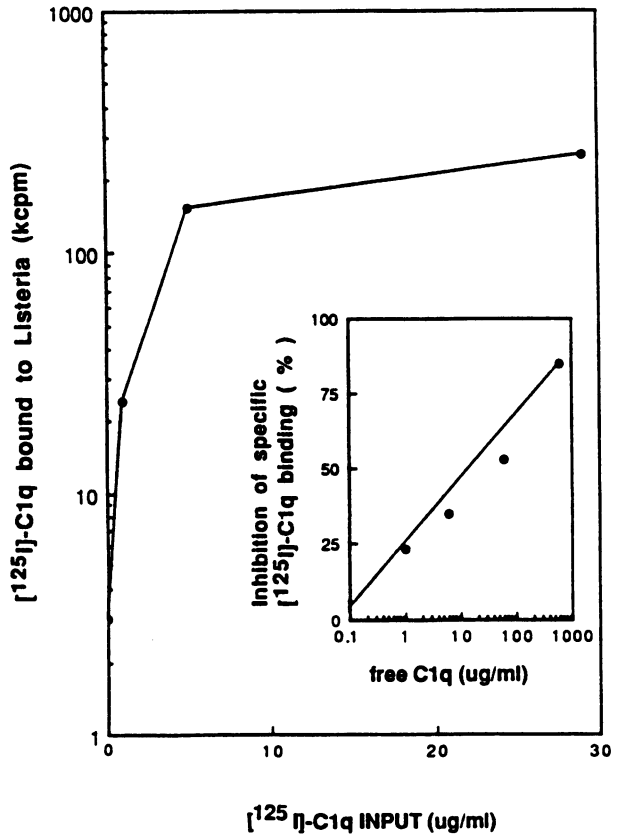

FIG. 4. Binding of ^{125}I-C1q to *L. monocytogenes*. Bacteria, 10^7, were incubated with various amounts of ^{125}I-C1q. (Inset) Competition assay using a fixed amount of ^{125}I-C1q (8 μg/ml) and variable amounts of nonlabeled C1q.

at 4°C (temperature at which receptor-mediated endocytosis does not occur), the phagocytosis of C1q-opsonized *L. monocytogenes* by untreated Mϕ-like cell lines was comparable to that observed with a combination of unopsonized *L. monocytogenes* and cells in the presence of excess C1q (4.8 × 10^5 versus 5.1 × 10^5 CFU, respectively) (B versus C bars in Fig. 7, left panel). Hence, preincubation of Mϕ with saturating amounts of C1q at 37°C (G bars) inhibited the uptake observed at 4°C (C bars) to a level comparable to that observed with both untreated cells and unopsonized *L. monocytogenes* (A and E bars at 4 and 37°C, respectively).

DISCUSSION

In this report, a model for C1q-related uptake of *L. monocytogenes* by Mϕ is described. Initial experiments demonstrated that *L. monocytogenes* uptake was increased when the bacteria were previously opsonized with normal serum, and this effect was abrogated upon heat inactivation of serum. To study the role of complement components other than C3 (a role for C3 and CR3 in *L. monocytogenes* phagocytosis has been described recently [9]), our attention was focused on the role of C1q. C1q was considered a strong candidate for study because of its presence in serum and in other fluids at relatively high concentration and because it mediates enhanced ingestion of other pathogens (4, 41). A clear role for C1q in the uptake of *L. monocytogenes* by Mϕ was demonstrated in several experiments performed to test this hypothesis. Data showed that (i) C1qDS-opsonized *L. monocytogenes* cells were taken up by Mϕ in the same range as *L. monocytogenes* in a serum-free medium (R0) and much less than NHS-opsonized *L. monocytogenes*; (ii) purified C1q, used as an opsonin, notably enhanced *L. monocytogenes* uptake; (iii) this effect was selectively blocked by anti-C1q F(ab')$_2$ antibody fragments; (iv) FITC-labeled C1q was specifically localized on *L. monocytogenes*; and (v) radioiodinated C1q demonstrated the saturable nature of the binding to *L. monocytogenes*. Intracellular pathogens, in most cases, have been reported to enter host cells through cell surface receptors. In this regard, complement receptors

TABLE 1. Inhibition of ^{125}I-C1q binding to *L. monocytogenes* by cell wall constituents[a]

Expt	Competitor added	Inhibition (cpm) with given amt of competitor			
		None	0.2 μg/ml	2 μg/ml	20 μg/ml
1	None	130,000			
	Unlabeled C1q		100,100 (23)[b]	71,500 (45)	55,943 (55)
	Collagen type I		123,500 (5)	123,280 (6)	123,271 (6)
	D-Galactose		123,600 (5)	122,890 (5.5)	122,813 (5.5)
	D-Mannose		123,700 (5)	123,600 (5)	123,133 (6)
2	None	22,392			
	Unlabeled C1q		ND	ND	9,716 (56)
	MDP (L-D)		19,481 (13)	16,794 (25)	13,042 (42)
	MDP (D-D)		20,600 (8)	20,152 (10)	20,104 (11)
	MDP (L-L)		ND	ND	19,786 (11)
	D-Galactose		22,220 (5)	22,200 (5)	22,198 (5)
	D-Mannose		20,830 (7)	20,824 (7)	19,343 (11)
3	None	25,460			
	Unlabeled C1q		ND	ND	11,457 (55)
	DAP		22,404 (12)	19,858 (22)	14,846 (42)
	Muramic acid		22,914 (10)	21,131 (17)	18,363 (18)
	L-Rhamnose		23,000 (10)	19,095 (25)	14,870 (40)

[a] ^{125}I-C1q (2 μg/ml) was incubated with a fixed amount of *L. monocytogenes* (10^7 bacteria per ml) (see Materials and Methods for details). ND, not done; MDP (*x*-*x*), *N*-acetyl-muramyl-dipeptide isoforms; DAP, diaminopimelic acid.

[b] Value in parentheses is percentage of inhibition.

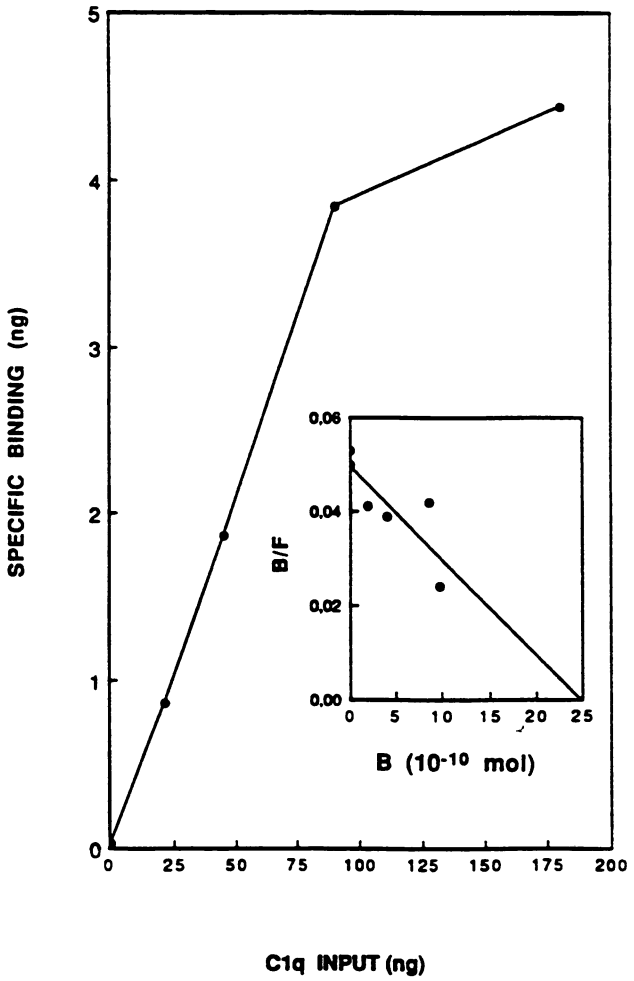

FIG. 5. Number of C1q binding sites and binding affinity on IC-21 Mϕ. Cells, 10^6, were incubated with increasing amounts of ^{125}I-C1q. Results have been corrected for nonspecific binding as described in Materials and Methods. (Inset) Scatchard analysis. B, bound C1q; F, free C1q. Data show 102,000 binding sites per cell ($K_a = 2.09 \times 10^7\ M^{-1}$).

can be used by pathogens to facilitate their entry into cells. Attachment to C3 receptors has been reported for *Leishmania* spp. (48) that gain the intracellular environment through CR3 or bacteria such as *Legionella pneumophila*, *M. tuberculosis*, and *M. leprae* that are able to attach to cells through CR1, CR3, and/or CR4 (30, 37, 38). With regard to *L. monocytogenes*, it has been reported recently that its ability to fix C3 facilitates its uptake through CR3 on phagocytes (9).

A role for C1q in the uptake of *T. cruzi* (34) and *S. minnesota* (35) by mammalian cells has been described. In addition, binding structures for C1q were reported in *Schistosoma mansoni* (36). The next experiments addressed the question of whether C1qRs played a significant role in the internalization of C1q-opsonized *L. monocytogenes* by Mϕ. C1q receptors have been described in Mϕ (10) and in other cells of myeloid lineage (8, 13, 28, 31, 43, 44). Due to the wide heterogeneity of cells able to bind C1q, a unique C1qR protein has not been found. Several laboratories have reported the isolation of C1q binding proteins from different cells (8, 10, 14, 31, 43, 44). While all of these glycoproteins are acidic in nature, they have significantly different biochemical characteristics. Recently, it has been reported that one of the proteins in the C1qR complex, at least in human leukocytes, is CD43 (sialophorin/leukosialin) (14), which shows great receptor complexity. In this regard, the presence of C1q receptors on the Mϕ-like cells used in this study is demonstrated. The number of binding sites and the binding affinity constants were similar to those reported in other Mϕ-like cell lines: 4.25×10^5 receptor sites per cell with a K_a of $8.1 \times 10^6\ M^{-1}$ in the case of WEHI-3 (10), and 1.02×10^5 receptor binding sites per cell with a K_a of $20.9 \times 10^6\ M^{-1}$ in the case of IC-21 (this report). Although a monoclonal antibody against the Raji cell-derived C1q binding protein that mimics C1q-mediated inhibition of collagen-induced platelet aggregation has been described (31), an antibody able to block or mimic the C1q-mediated effects on phagocytic cells has not been found (14). Therefore, to study the role of this receptor in the ingestion of C1q-opsonized *L. monocytogenes*, purified C1q at a concentration able to saturate the C1q binding structures of Mϕ was used. It was

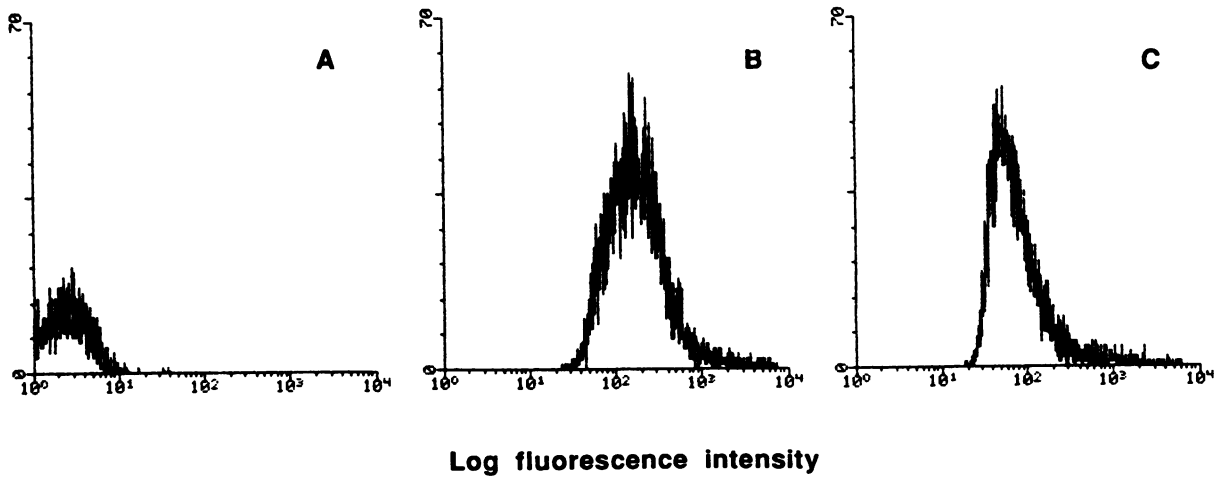

FIG. 6. FITC-C1q staining of IC-21 cells blocked by unlabeled C1q. In this experiment, IC-21 cells were incubated with FITC-C1q in the absence (B) or presence (C) of a saturating amount of unconjugated C1q. A profile of unlabeled cells is shown in panel A. Cells were analyzed by fluorescence-activated flow cytometry, and the results are displayed as histograms on a four-decade logarithmic scale. Events measured per sample were 2,468 (A), 8,535 (B), and 6,725 (C). Peak channels were 1, 153.9, and 54.2 in panels A, B, and C, respectively.

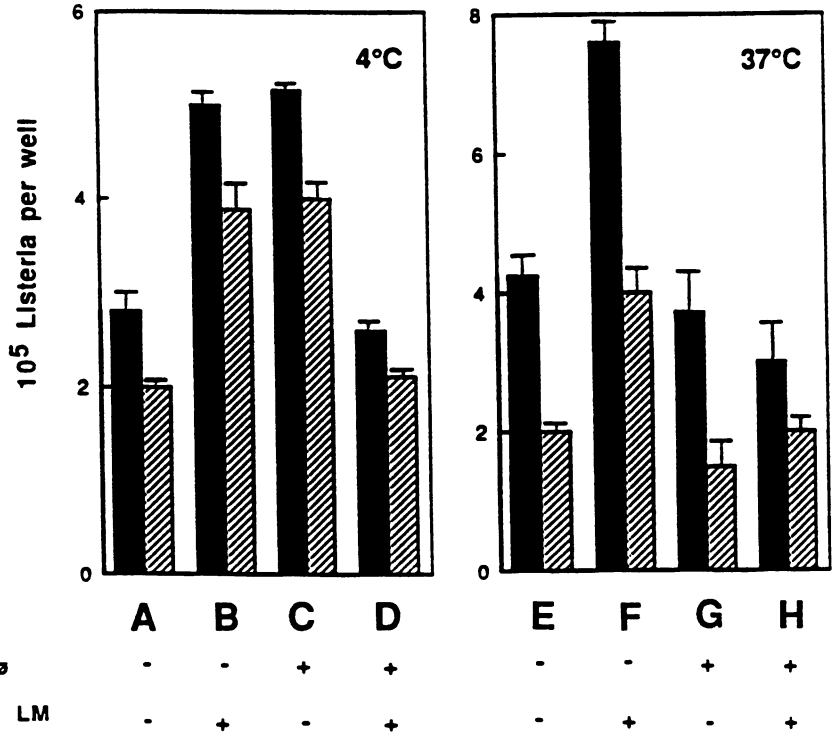

FIG. 7. Pretreatment of host cells with C1q decreases ingestion of C1q-opsonized *L. monocytogenes*. Bacteria (2×10^6), preopsonized with C1q (100 μg/ml) or not preopsonized, were added to four different sets of Mϕ-like cells. They were incubated with (60 μg/ml) or without C1q for 60 min at 4°C (left panel) or 37°C (right panel). Results are expressed as mean CFU ± SD of triplicate experiments. Statistical significance between experimental groups was as follows: A versus B ($P < 0.005$); A versus C ($P < 0.005$); E versus F ($P < 0.005$); F versus G ($P < 0.005$). Filled bars, IC-21 cells; striped bars, P-388D1 cells.

observed that enhanced uptake of C1q-opsonized *L. monocytogenes* did not occur when Mϕ were pre-incubated with saturating concentrations of purified C1q. These data suggest that C1q receptors (or more properly, C1q binding structures) on Mϕ mediate most of the phagocytosis of C1q-treated *L. monocytogenes*. To minimize the effects caused by other molecules, the assay was performed in a serum-free system. C1q fixation to bacteria has been well studied in gram-negative bacteria, and it has been postulated that the acceptor structure for C1q attachment is focused on lipopolysaccharide molecules (25, 26). These studies showed that the interaction is dependent on the lipid A portion of the molecule. However, with gram-positive bacteria, very few data regarding their interaction with complement components have been reported, probably because it is generally assumed that these bacteria do not activate the classical pathway of complement, and complement does not play a role in the direct killing of gram-positive bacteria (7, 27). It was of interest to attempt to characterize the chemical structures in the *L. monocytogenes* cell wall responsible for C1q attachment. Inhibition of the C1q-*L. monocytogenes* binding was observed with two *L. monocytogenes* peptidoglycan constituents, *N*-acetyl-muramyl-dipeptide (L-D isoform) and diaminopimelic acid, as well as with L-rhamnose. Other peptidoglycan components such as muramic acid or other *N*-acetyl-muramyl-dipeptide isoforms (L-L or D-D) did not inhibit C1q binding. C1q binding has carbohydrate specificity, and it is effective with L-rhamnose, a saccharide reported previously to be the immunologically active carbohydrate of *L. monocytogenes* serotype 1 (46), and is not effective with D-galactose or D-mannose, both found only at very low concentrations in this serotype. These data indicate that the C1q binding structure on *L. monocytogenes* is the cell wall skeleton. That human type I collagen did not inhibit ^{125}I-C1q binding to *L. monocytogenes* does not indicate the involvement of the collagenlike portion of C1q in the attachment to *L. monocytogenes*. Structures of other bacteria able to bind C1q have also been described. Phenolic glycolipid-1 of *M. leprae* avidly binds C1q (39). This binding requires both the terminal trisaccharide and the mycocerosyl fatty acyl side chains. In addition, C1qR is known to bind to the collagenlike region of C1q (2). These data, together with the observation that C1q binding to polysaccharides has chain length specificity (binding is more effective with three saccharide moieties) (40), support the idea that the globular portion of C1q binds to sugar chains on the bacterium cell wall skeleton. Receptor-mediated association of C1q with their target cells leads to a variety of cellular responses (12), including enhancement of phagocytosis, stimulation of oxidative metabolism leading to production of damaging oxygen radicals (45), enhancement of antibody-dependent and -independent cell cytotoxicity (5, 16, 21–23), and inhibition of interleukin-1 expression (15). Nevertheless, what has been reported for C3 receptors shows that entry through CR3 provides the intracellular pathogens with safe passage to host cells (37, 38, 48). However, ligand-receptor interactions may influence intracellular events such as the change in the order in which granule components are incorporated into the phagosome, as has been reported when *S. typhimurium* is opsonized with C3 or IgG (19). However, it seems that not all routes of complement receptors lead to activation of Mϕ and killing of bacteria, as in the case of CR3 (39).

It is clear that, in spite of the roles of C3/CR3 (9) and C1q/C1qRs (this report) in *L. monocytogenes* phagocytosis by Mϕ, other mechanisms mediating *L. monocytogenes* internalization in mammalian cells should exist. The uptake of *L. monocytogenes* by cells in serum-free systems favors this hypothesis. In fact, several nonopsonic forms of recognition between phagocytes and microorganisms have been described (for a review, see reference 29). In the particular case of *L. monocytogenes*, a 60-kDa protein which is essential for mouse fibroblast invasion has been reported (20). It has also been reported that entry of *L. monocytogenes* into epithelial cells is mediated by an 80-kDa protein, internalin, a repeat protein similar to the surface proteins of gram-positive cocci (11). It is probable that other molecules specifically related to microbe-host cell interplay in *L. monocytogenes* or in mammalian cells or in both will be described in the future.

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