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Roles of Complement and Complement Receptor Type 3 in Phagocytosis of *Listeria monocytogenes* by Inflammatory Mouse Peritoneal Macrophages

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***Listeria monocytogenes* is a facultative intracellular bacterium that is phagocytosed by and can proliferate within cells of the mononuclear phagocyte system. However, the receptors used by macrophages to internalize this organism have not been identified. In the experiments described here, the contributions of serum complement component C3 and macrophage complement receptor type 3 (CR3) to opsonization and phagocytosis of *L. monocytogenes* by mouse inflammatory peritoneal macrophages were studied. An assay which allowed the distinction of adherent versus internalized bacteria was used to show that following mixing of *L. monocytogenes* with inflammatory macrophages, greater than 95% of the bacteria bound were internalized by these phagocytes. When immunofluorescent antibodies to C3 and immunoglobulin were used, C3 but not immunoglobulin was detected on *L. monocytogenes* following incubation in normal serum or ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetracetic acid-Mg^{2+}-chelated serum. When macrophages were incubated with 5% serum and *L. monocytogenes* in a standard assay, approximately 80% of the phagocytosis was inhibited by heat-inactivated serum or by the addition of $F(ab')_2$ anti-C3 antibody. The role of macrophage CR3 was demonstrated by the ability of anti-CR3 monoclonal antibody M1/70 to decrease phagocytosis to the same levels as those seen with heat-inactivated serum. These experiments indicated that in the presence of normal serum, *L. monocytogenes* is phagocytosed by inflammatory macrophages primarily because CR3 on these cells binds to C3 deposited on the bacterial surface.**

When a host is confronted by an invading organism, one of the earliest and most effective host defense mechanisms is the inflammatory reaction. This response is the summation of fluid-phase and cellular processes acting in concert to contain and eliminate the pathogen. The complement system is an important part of inflammation and is able to focus this generalized event to promote specific host effector functions, such as chemotaxis, opsonization, and phagocytosis. This channeling is in part accomplished by receptors on leukocytes which specifically recognize certain complement proteins or their proteolytic fragments. In this way, the immune system can operate in a more directed and efficient manner and rapidly respond to a wide range of pathogens. The important contributions of complement and complement receptors (CRs) to host defense are clearly illustrated by the profound and deleterious consequences created by their absence (1, 48).

Complement and CRs may be used not only for host defense but also by a pathogen to its own advantage. For example, various organisms might utilize CRs to facilitate their entry into host cells in a way that allows them to avoid the untoward events following phagocytosis and to use the intracellular environment as a haven from host defenses (35). Among such organisms are obligate intracellular pathogens, such as *Leishmania* species and *Histoplasma capsulatum* (8, 34, 59). Facultative intracellular bacteria may also use CRs in this fashion. These include *Legionella pneumophila*, *Mycobacterium tuberculosis*, and *M. leprae*, each of which enters human mononuclear phagocytes via CR types 1 (CR1) and 3 (CR3) (42, 52, 53). Furthermore, a role for the C1q receptor in the entry of the Re mutant of *Salmonella minnesota* into pulmonary endothelial cells has also been suggested (50).

Listeria monocytogenes is a facultative intracellular bacterium which has been used as a model to study cell-mediated immune responses and inflammation. In natural infections, *L. monocytogenes* presumably enters the host through the gastrointestinal tract, traverses the epithelial lining, and then spreads systemically (18, 29). In murine infections, it accumulates in the liver and spleen, where it is able to replicate in the mononuclear phagocyte system as well as in other cells, such as hepatocytes (33, 42). Recovery from infection appears to depend, at least in part, upon T-cell-mediated recruitment of listericidal inflammatory phagocytes to foci of infection (12, 30, 39). Early in the course of this response, neutrophils are the predominant inflammatory cells, but they are replaced in 48 to 72 h by blood- and bone marrow-derived mononuclear phagocytes (31, 39). It is these latter cells which ultimately become the major effector cells of listerial immunity. However, the receptors used by these listericidal macrophages to internalize this bacterium have not been studied.

Earlier reports suggested that *L. monocytogenes* is an activator of the alternative pathway of complement and that heat-labile serum factors, presumably complement, are important opsonins for this bacterium (2, 21, 57, 58). In this study, we define the roles of serum complement component C3 and CR3 on mouse inflammatory macrophages in the opsonization and phagocytosis of *L. monocytogenes*. Three main questions were addressed. (i) Following binding of *L. monocytogenes* to inflammatory phagocytes, are the bacte-

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ria internalized or do they remain extracellular? (ii) Is complement component C3 an important opsonin of *L. monocytogenes* during incubation with normal mouse serum? (iii) Does CR3 play a significant role in the binding and phagocytosis of complement-opsonized *L. monocytogenes* by mouse macrophages?

MATERIALS AND METHODS

Mice. (C57BL/6 × DBA/2)F_1 (BDF_1) mice were obtained from Jackson Laboratory, Bar Harbor, Maine. Animals were housed in the National Jewish Center for Immunology and Respiratory Medicine Animal Care Facility, fed mouse chow, and given water ad libitum. Mice of either sex were used at 8 to 16 weeks of age.

Sera. Normal mouse serum (NMS) was obtained from Swiss-Webster mice. The mice were exsanguinated by cardiac puncture under chloroform anesthesia, and whole blood was allowed to clot for 60 min on ice and centrifuged at 1,000 × *g* for 20 min at 4°C. Serum was removed, divided into 0.5-ml aliquots, and stored at −70°C. An aliquot was thawed immediately prior to use in each experiment.

Bacteria. *L. monocytogenes* EGD was maintained in a virulent state by periodic passage in BDF_1 mice, and log-phase cultures were stored at −70°C in 0.5-ml aliquots. Prior to use, an aliquot was thawed and incubated overnight in 5 ml of tryptose phosphate broth (Difco Laboratories, Detroit, Mich.) at 37°C with constant agitation. For each experiment, the bacterial concentration was determined by counting in a Petroff-Hausser counting chamber.

Cells. Peritoneal exudate cells (PECs) were obtained by injecting mice intraperitoneally with 1.0 ml of sterile 10% proteose peptone (Difco). The cells were harvested 48 h later by peritoneal lavage with 10 ml of cold, sterile balanced salt solution (BSS) (33) or 0.01 M phosphate-buffered saline (PBS) (pH 7.2) without added divalent cations. The cells were centrifuged at 250 × *g* for 10 min, resuspended in either BSS or PBS, and counted in a hemacytometer. As previously described, peritoneal exudates obtained in this manner consisted of 85 to 90% macrophages, 5 to 10% neutrophils, and <5% lymphocytes (9).

Deposition and detection of C3 on *L. monocytogenes*. Following overnight growth, *L. monocytogenes* was pelleted at 12,500 × *g* for 5 min, washed three times with PBS, and resuspended to the original volume in PBS. Then, 10^8 bacteria were incubated with 20% NMS, 20% heat-inactivated mouse serum (HIMS), prepared by heating serum at 56°C for 30 min, or 20% NMS with 10 mM EDTA or 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid–7 mM $MgCl_2$ (EGTA-Mg^{2+}). The tubes were rotated at 37°C for 30 min and centrifuged at 12,500 × *g* for 5 min, and the bacteria were washed twice with PBS. To duplicate tubes was added 20 μg of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse C3, which does not discriminate between C3b and iC3b, per ml or a 1:60 dilution of polyclonal FITC-labeled goat anti-mouse immunoglobulin (Ig). In some experiments, a 1:50 dilution of polyclonal lissamine rhodamine B (LRSC)-labeled goat anti-mouse IgG-IgM was used. After 20 min of incubation at 25°C, the bacteria were pelleted, washed twice with PBS, resuspended in 40 μl of distilled H_2O, placed on glass microscope slides, and allowed to air dry. Slides were examined by fluorescence microscopy (Leitz, Wetzlar, Germany) under oil immersion (magnification, ×1,000). Bacteria incubated in serum were compared with bacteria incubated in PBS alone as negative controls. C3 or Ig deposition was defined by the presence of specific staining indicated by circumferential fluorescence of the target at a titer of FITC-labeled antibody which did not cause an increase in the background autofluorescence of unopsonized bacteria. Positive controls for anti-mouse Ig consisted of mouse spleen cells and *L. monocytogenes* incubated in mouse anti-*L. monocytogenes* serum.

To quantitate the binding of C3 and Ig to *L. monocytogenes* after incubation with serum and fluorochrome-labeled antibodies, we resuspended bacteria in 2.0 ml of PBS and evaluated them for immunofluorescence on a Hitachi F-4010 fluorescence spectrophotometer. Excitation and emission wavelengths were 494 and 520 nm for FITC and 570 and 590 nm for LRSC, respectively.

Assays for uptake and phagocytosis of *L. monocytogenes*. The ability of PECs to take up *L. monocytogenes* was measured with a standard assay as described previously (11). In brief, 2.5 × 10^6 PECs and 2.5 × 10^7 *L. monocytogenes* were mixed with 5% NMS and diluted to a 1-ml final volume in polypropylene snap-cap tubes (12 by 75 mm; Falcon; Becton-Dickinson Labware, Lincoln Park, N.J.). The tubes were rotated end-over-end for 30 min at 37°C and centrifuged at 300 × *g* for 10 min at 4°C. Free bacteria were removed by three washes with 2.0 ml of iced BSS. The cells were resuspended in 1.0 ml of PBS containing 5% fetal calf serum (FCS), and 0.1 ml was removed to make cytocentrifuge slides, which were fixed, stained with Diff-Quik (AHS del Caribe, Aquado, P.R.), and examined by light microscopy under oil immersion (magnification, ×1,000). Uptake is expressed by convention as the phagocytic index (PI), calculated as the percentage of macrophages containing one or more bacteria times the mean number of bacteria per positive cell.

Evaluation of bacterial uptake by staining and light microscopy as described above does not allow discrimination between bacteria adherent to and bacteria internalized by phagocytes. Therefore, to determine accurately whether PEC-associated *L. monocytogenes* were intracellular or bound to the extracellular surface of the phagocytes, we used a modification of the assay which measures bacterial uptake. This method is described in detail in reference 14. Heat-killed *L. monocytogenes* (56°C for 60 min) were labeled with FITC by the method of Gelfand et al. (17) by incubation with 0.1 mg of FITC isomer 1 (Sigma Chemical Co., St. Louis, Mo.) per ml in 0.1 M $NaHCO_3$ (pH 9.0) at 25°C for 60 min. Alternatively, live *L. monocytogenes* were labeled with 0.1 mg of FITC per ml in PBS (pH 7.2) at 37°C for 2 h. In both cases, bacteria were pelleted at 12,500 × *g* for 5 min and washed free of unbound FITC with PBS. Live, labeled bacteria were stored frozen at −70°C and thawed immediately prior to use in the assay. PECs and labeled bacteria were mixed in a 1:10 ratio in the presence of 5% NMS, HIMS, or diluent only, and the tubes were rotated end-over-end for 30 min at 37°C. The cells were washed three times with 2.0 ml of iced BSS and resuspended in PBS with 5% FCS. Next, 95-μl aliquots of PECs were removed and mixed with ethidium bromide (50-μg/ml final concentration), and a 10-μl drop was immediately placed on a glass slide, overlaid with a coverslip, and evaluated by fluorescence microscopy under oil immersion.

In certain experiments in the uptake assay, HIMS or no serum was used. In experiments in which monoclonal antibodies were used to inhibit phagocytosis, the antibody was mixed with cells, serum, and BSS, and the mixture was incubated on ice for 30 min prior to the addition of bacteria. For confirmation that the M1/70 $F(ab')_2$ fragments (described below) did not inhibit the phagocytosis of IgG-coated

targets, as reported by others (4, 38), *L. monocytogenes* was opsonized in heat-inactivated mouse anti-*L. monocytogenes* serum and added to PECs treated with 3 μg of $F(ab')_2$ fragments per ml. Incubation in the presence of the antibody did not inhibit the phagocytosis of these bacteria (data not shown).

Antibodies. The B-cell hybridomas M1/70, which secretes a rat IgG2b antibody specific for mouse CR3 (4), R17,217, which secretes a rat IgG2a anti-mouse transferrin receptor antibody (28), and 11B11, which secretes an IgG1 anti-interleukin-4 antibody (41), were obtained from the American Type Culture Collection. As a control, RIgS-2b, a rat IgG2b antibody directed against an unknown epitope, was purchased from PharMingen, San Diego, Calif. M1/70 was cultured in RPMI 1640 (Irvine Scientific, Santa Ana, Calif.) with 5% FCS–0.05 mM 2-mercaptoethanol–2 mM L-glutamine–50 μg of gentamicin per ml. Culture supernatant was passed over a protein G-Sepharose 4 Fast Flow (Pharmacia Fine Chemicals, Piscataway, N.J.) column and eluted with 0.1 M acetic acid (pH 2.8). $F(ab')_2$ fragments were produced by digestion of the purified IgG with 5% pepsin (Sigma) at pH 4.5 for 18 h at 37°C with agitation. $F(ab')_2$ fragments were separated from undigested IgG with a 600-ml Sephadex G-150 Superfine (Pharmacia) column (7). Fractions were assessed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with standard Coomassie blue staining (20, 27). Fractions found to be free of intact IgG were pooled, dialyzed against PBS, and used at various concentrations. R17,217 was cultured in DMEM (GIBCO, Grand Island, N.Y.) with a high concentration of glucose–2% FCS–0.05 mM 2-mercaptoethanol–2 mM L-glutamine–50 μg of gentamicin per ml. 11B11 was cultured in RPMI 1640 with 3% FCS–0.05 mM 2-mercaptoethanol–2 mM L-glutamine–50 μg of gentamicin per ml. The IgG fractions were collected by passage over a protein G-Sepharose 4 Fast Flow column, concentrated, dialyzed against PBS, and frozen at −20°C in aliquots until used. All concentrations were determined by measuring the optical density at 280 nm. For fluorescence staining of opsonized bacteria, commercial FITC-labeled antibodies were obtained. FITC-labeled $F(ab')_2$ fragments of polyclonal goat anti-mouse C3 were purchased from Cappel Research Products, Organon Teknika (Durham, N.C.), FITC-labeled polyclonal goat anti-mouse Ig was purchased from Sigma, and polyclonal LRSC-labeled goat anti-mouse IgG-IgM was purchased from Jackson ImmunoResearch Laboratories, West Grove, Pa.

RESULTS

Discrimination between adherent and internalized *L. monocytogenes*. The purpose of the experiments described in this paper was to determine the role of C3 and CR3 in the phagocytosis of *L. monocytogenes* by macrophages. Therefore, it was necessary to measure accurately whether PECs which have taken up *L. monocytogenes* have indeed internalized the bacteria or whether the bacteria have merely adhered to the cells. To this end, we used ethidium bromide, FITC-labeled *L. monocytogenes*, and fluorescence microscopy.

Ethidium bromide is a vital dye which is largely excluded by live cells, which fluoresces a very bright red-orange, and which causes FITC-labeled targets to shift from green to red fluorescence (15). When added to PECs which have taken up FITC-labeled bacteria, ethidium bromide causes extracellularly attached bacteria to fluoresce red-orange, whereas internalized bacteria remain green. This technique is described in detail elsewhere (14).

TABLE 1. Discrimination between adherent and internalized *L. monocytogenes* following binding to inflammatory peritoneal macrophages[a]

Bacteria[b]	Material that bacteria were incubated with:	No. of:		% Internalized
		Total bacteria[c]	Internal bacteria[d]	
Heat killed	NMS	447	425	95.1
Heat killed	NMS + M1/70[e]	180	175	97.2
Live	NMS	287	274	95.5
Live	HIMS	146	142	97.3
Live	BSS	149	144	96.6

[a] PECs were mixed with FITC-labeled bacteria in 5% NMS, 5% HIMS, or BSS for 30 min at 37°C. The cells were washed and resuspended in PBS with 50 μg of ethidium bromide per ml and examined by fluorescence microscopy under oil immersion.
[b] Heat-killed *L. monocytogenes* was labeled with 0.1 mg of FITC per ml in 0.1 M $NaHCO_3$ (pH 9.0). Live bacteria were labeled with FITC in PBS (pH 7.2).
[c] Bacteria from 25 consecutive PECs with 1 or more bacteria per cell were counted.
[d] Determined by red-green color discrimination.
[e] M1/70 $F(ab')_2$ fragments were used at 10 μg/ml.

Using fluorescence microscopy, we conducted a quantitative determination of adherent versus internalized bacteria under various opsonizing conditions with live and heat-killed *L. monocytogenes* (Table 1). We found that under all conditions tested, greater than 95% of cell-associated *L. monocytogenes* were internalized and not merely adherent. The high percentage of internalization was true for both live and heat-killed bacteria. These data indicate that inflammatory mouse peritoneal macrophages internalize nearly all cell-associated *L. monocytogenes*.

Deposition of complement on *L. monocytogenes*. To test whether C3, Ig, or both were deposited on bacteria following incubation in NMS, we used fluorescein-labeled anti-C3 and anti-Ig antibodies. As illustrated in Table 2, *L. monocytogenes* incubated in NMS had easily detectable fluorescence when the bacteria were incubated with FITC-labeled anti-C3 antibody, but no specific fluorescence was detected following incubation in HIMS or diluent alone. To determine whether C3 fragments were deposited by the alternative complement pathway, bacteria were incubated in NMS containing either 10 mM EDTA, which blocks both classical and alternative pathways, or EGTA–Mg^{2+}, which blocks

TABLE 2. Deposition of C3 on *L. monocytogenes*

Material that bacteria were incubated with[a]:	Result of:	
	Fluorescence microscopy[b]	Fluorometry[c]
NMS	+	53.0
HIMS	−	3.1
PBS	−	2.1
NMS + 10 mM EDTA	−	2.3
NMS + EGTA-Mg^{2+}	+	67.2

[a] Bacteria (10^8) were incubated in 20% NMS, HIMS, diluent only (PBS), or NMS with chelating agents as indicated, washed, incubated with FITC-labeled anti-C3 antibody, washed again, and examined by fluorescence microscopy under oil immersion and fluorescence spectrophotometry.
[b] +, specific staining detected as concentric linear fluorescence of the cell wall; −, no staining.
[c] Relative fluorescence intensity measured at 520 nm.

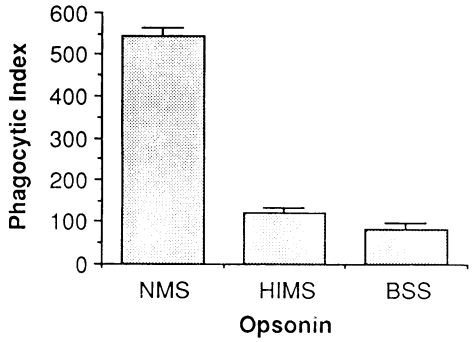

FIG. 1. Phagocytosis of *L. monocytogenes* as a function of opsonin. PECs and bacteria were mixed with 5% NMS, 5% HIMS, or BSS alone for 30 min at 37°C. Results are expressed as the mean PI ± the standard error of the mean for four separate experiments.

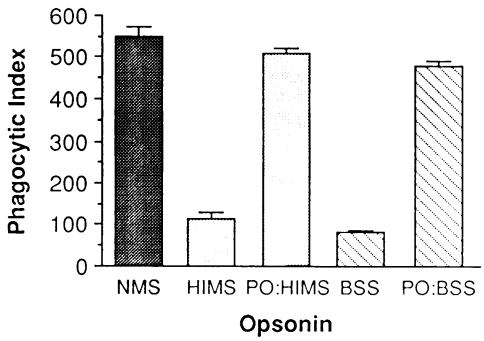

FIG. 2. Phagocytosis of preopsonized *L. monocytogenes*. Bacteria were incubated in NMS, washed, and mixed for 30 min with PECs in either 5% HIMS (PO:HIMS) or diluent only (PO:BSS), and the PI was determined. These bacteria were compared with simultaneously assayed, unopsonized bacteria mixed with PECs in 5% NMS, 5% HIMS, or BSS only. Results are expressed as the mean PI ± the standard error of the mean for two (BSS and PO:BSS) or three (HIMS and PO:HIMS) separate experiments.

only the classical pathway (13). As shown in Table 2, *L. monocytogenes* incubated with EDTA showed no deposition of C3 fragments; however, specific fluorescence was clearly seen when the bacteria were incubated with EGTA-Mg^{2+}, indicating the deposition of C3 fragments by the alternative pathway. Results of fluorescence microscopy and fluorescence spectrophotometry were the same (Table 2).

A similar procedure was used to test for the presence of Ig on *L. monocytogenes*. With FITC-labeled anti-Ig antibody and fluorescence microscopy, Ig was not detected on the bacteria after they were incubated in 20% NMS. Positive controls included mouse spleen cells and *L. monocytogenes* incubated in heat-inactivated mouse anti-*L. monocytogenes* serum, the latter demonstrating Ig deposition at titers of 1:1 to 1:512. These results were confirmed with fluorescence spectrophotometry. LRSC-labeled anti-Ig antibody demonstrated the deposition of Ig when bacteria were incubated in antiserum at titers of 1:50 and 1:500 but not when they were incubated in 20% NMS (data not shown).

Contribution of C3 to the phagocytosis of *L. monocytogenes*. For an initial test of the contribution of serum complement to the phagocytosis of *L. monocytogenes*, the phagocytosis assay was performed in the presence of NMS, HIMS, or the diluent BSS only (Fig. 1). The data showed that the majority of phagocytosis, 78%, was abrogated by heat inactivation of the serum. Moreover, there was no difference between phagocytosis in the presence of HIMS or BSS, suggesting that antibody played little, if any, role.

Further experiments were conducted to determine whether preincubation in NMS would allow phagocytosis to occur when cells and bacteria were incubated with HIMS or without serum. *L. monocytogenes* was preopsonized in 20% NMS, pelleted at 12,500 × *g*, and washed three times with BSS to remove serum. These bacteria were used in the phagocytosis assay in the presence of either 5% HIMS or diluent only. As shown in Fig. 2, phagocytosis of preopsonized *L. monocytogenes* in HIMS or without serum proceeded essentially as well as when PECs and bacteria were incubated in NMS.

Next, experiments were conducted to evaluate more specifically the contribution of complement component C3 to phagocytosis. For these experiments, the phagocytosis assay with 5% NMS was performed in the presence of 100 μg of $F(ab')_2$ fragments of anti-C3 antibody per ml. As shown in Fig. 3, this antibody reduced the PI to approximately 15% of the control, presumably by preventing the deposition of C3 fragments on the bacterial surface and/or by interfering with the ability of deposited C3 fragments to interact with complement receptors on the PECs.

Contribution of CR3 to the phagocytosis of *L. monocytogenes*. To test the hypothesis that CR3 on macrophages has a significant role in the phagocytosis of opsonized *L. monocytogenes*, we used M1/70, a rat anti-mouse CR3 antibody. PECs were preincubated with various concentrations of M1/70 IgG on ice for 30 min, and the phagocytosis of *L. monocytogenes* in 5% NMS was tested. As shown in Fig. 4, M1/70 IgG caused a dose-dependent inhibition of phagocytosis. None of the control antibodies inhibited phagocytosis (Table 3).

M1/70 IgG caused the aggregation of PECs during the assay, as also reported by Rosen and Gordon (44). Because it seemed likely that this clumping could inhibit the phagocytic ability of the PECs independent of the CR3 blockade, giving false-positive results, $F(ab')_2$ fragments were made and tested. When either unseparated fragments, containing a very small amount of IgG, as estimated by SDS-PAGE, or fragments purified by column chromatography were used in

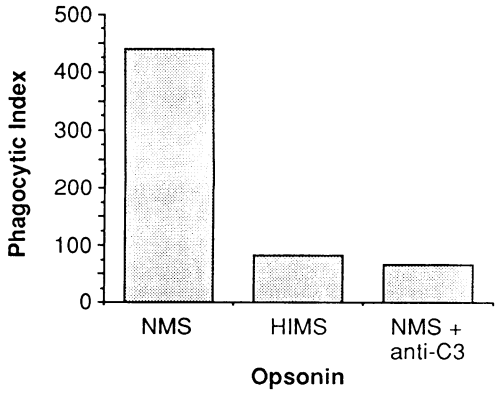

FIG. 3. Ability of anti-C3 antibody to inhibit the phagocytosis of *L. monocytogenes* by inflammatory peritoneal macrophages. Bacteria and PECs were mixed in 5% NMS, 5% HIMS, or 5% NMS with 100 μg of $F(ab')_2$ fragments of polyclonal anti-mouse C3 antibody per ml. Cells and bacteria were incubated for 30 min at 37°C, and the PI was determined. The results of one of three similar experiments are shown.

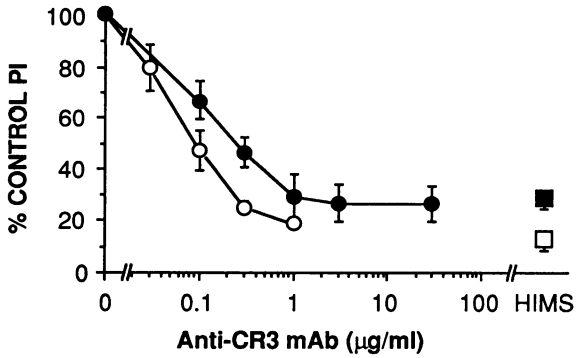

FIG. 4. Ability of anti-CR3 antibody (mAb) to inhibit the phagocytosis of *L. monocytogenes* by mouse inflammatory macrophages. PECs were preincubated for 30 min with the indicated concentrations of M1/70 IgG (○) or F(ab')$_2$ fragments (●) in 5% NMS or with 5% HIMS without antibody (□ and ■). Bacteria were added and incubated with PECs for 30 min, and the PI was determined. Inhibition of phagocytosis is expressed as the mean PI ± the standard error of the mean for control PECs and bacteria incubated in 5% NMS alone. Pooled results of three [F(ab')$_2$] or four (IgG) experiments are shown.

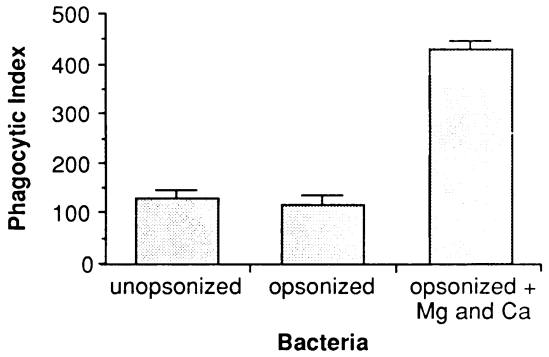

FIG. 5. Divalent cation requirement for the phagocytosis of opsonized *L. monocytogenes*. Bacteria were either unopsonized or opsonized in NMS, washed, and mixed with PECs. When used, divalent cations were added to a final concentration of 0.5 mM, and phagocytosis was assayed after 30 min. Results are expressed as the mean PI ± the standard error of the mean for three identical experiments.

the phagocytosis assay, the aggregation of PECs was not observed. The F(ab')$_2$ fragments inhibited phagocytosis in a dose-dependent fashion (Fig. 4), although a somewhat higher concentration was required to achieve maximal inhibition. In each case, phagocytosis could be decreased to the levels seen in HIMS. Phagocytosis in HIMS in these two cases was slightly different, probably because experiments were performed at different times with different lots of serum. All other experiments were performed with the same lot of serum as that used for the F(ab')$_2$ experiments. These data suggest that CR3 mediated the majority of the binding and phagocytosis of *L. monocytogenes* by inflammatory macrophages in NMS.

Divalent cation requirement for the phagocytosis of opsonized *L. monocytogenes*. The divalent cation requirement for the phagocytosis of opsonized *L. monocytogenes* was also evaluated. PECs were harvested in PBS without divalent cations, pelleted, and washed twice. They were mixed with unopsonized *L. monocytogenes* or with *L. monocytogenes* preopsonized in NMS. Phagocytosis was tested in PBS plus 5 mM glucose and with or without 0.5 mM Mg^{2+} and 0.5 mM Ca^{2+} (60). In the absence of divalent cations, there was no significant difference in the phagocytosis of opsonized and unopsonized bacteria (Fig. 5). The addition of Mg^{2+} and Ca^{2+} enhanced phagocytosis, indicating that efficient phagocytosis of opsonized bacteria requires these divalent cations.

TABLE 3. Specificity of the inhibition of the phagocytosis of *L. monocytogenes* by M1/70 F(ab')$_2$[a]

Hybridoma (μg/ml)	Subclass	PI
None		440
M1/70 F(ab')$_2$ (10)	Rat IgG2b	119
RIgS-2b (10)	Rat IgG2b	418
11B11 (10)	Mouse IgG1	416
R17,217 (13)	Rat IgG2a	431

[a] Antibody and PECs were preincubated for 30 min on ice prior to the addition of *L. monocytogenes*, and the PI was determined after 30 min.

DISCUSSION

The first experiments were conducted to evaluate critically whether *L. monocytogenes* is internalized by macrophages during a 30-min coculture in the presence of serum, a process later shown to be mediated in large part by CR3. An assay that discriminates extracellular from internalized bacteria on the basis of bacterial fluorescence demonstrated that 95% of *L. monocytogenes* incubated with inflammatory macrophages in the presence of NMS are indeed internalized and thus phagocytosed.

The second experiments used immunofluorescent-antibody staining to demonstrate that complement component C3 was deposited on the cell wall of *L. monocytogenes* following incubation in NMS. C3 was also deposited in the presence of EGTA-Mg^{2+}, presumably through activation of the alternative pathway of complement. This likely is a result of C3 binding to cell wall peptidoglycan. Other gram-positive bacteria have been reported to activate complement in this way, and it has been suggested that *L. monocytogenes* may do so as well (2, 24). Activation of the alternative pathway of complement by whole, heat-killed *L. monocytogenes*, as judged by the depletion of serum hemolytic activity in the presence of EGTA-Mg^{2+}, has previously been reported (57). We chose to demonstrate direct C3 deposition on the organism because spontaneous fluid-phase activation of the alternative pathway of complement in the absence of a stimulus can occur in the presence of EGTA-Mg^{2+}, giving false-positive results (13).

The opsonization of *L. monocytogenes* by complement plays a central role in the phagocytosis of this bacterium by mouse macrophages. When serum was heat inactivated, no appreciable C3 was deposited on the bacteria and phagocytosis was decreased to essentially the same levels as those seen in the absence of serum. Bacteria preincubated in NMS were phagocytosed well in either HIMS or diluent alone, suggesting that serum opsonins, presumably C3 fragments, bound to the bacterial cell wall were sufficient to mediate binding and phagocytosis. The ability of anti-C3 F(ab')$_2$ antibody fragments to block phagocytosis in NMS confirmed that C3 bound to *L. monocytogenes* mediated phagocytosis by peptone-elicited PECs.

The next question addressed was whether CR3 played a significant role in the phagocytosis of opsonized *L. monocy-*

togenes by PECs. CR3 is a leukocyte adhesion molecule, a member of the β_2 subgroup of the integrin superfamily of cell surface receptors, and is expressed in high numbers on mouse peritoneal macrophages (22, 45). It serves as the primary receptor for iC3b, and it is able to bind other ligands, such as lipopolysaccharide, β-glucan, and fibronectin (45). CR3 also serves as an important receptor on host mononuclear phagocytes for the binding and phagocytosis of several facultative intracellular bacteria, such as *M. tuberculosis*, *M. leprae*, and *Legionella pneumophila* (42, 52, 53). Given the importance of C3 bound to *L. monocytogenes* in the phagocytosis of this organism, it seemed reasonable to evaluate the contribution of macrophage cell surface CR3 to this process. When either intact IgG or $F(ab')_2$ fragments of anti-CR3 antibody were used, phagocytosis was inhibited to a level similar to that obtained with either heat-inactivated serum or 100 μg of anti-C3 antibody per ml. This result demonstrated that CR3 on mouse inflammatory peritoneal macrophages mediates most of the phagocytosis of *L. monocytogenes*.

The divalent cation requirement for the phagocytosis of opsonized *L. monocytogenes* was also tested. CR3 requires the divalent cations Mg^{2+} and Ca^{2+} to mediate binding and phagocytosis, whereas CR1 can function without them (60). In the absence of Mg^{2+} and Ca^{2+}, we found no difference in the phagocytosis of opsonized and unopsonized *L. monocytogenes*, indicating that the serum opsonins deposited on the bacteria require these cations to bind PEC surface receptors. The addition of 0.5 mM Mg^{2+} and Ca^{2+} to the system greatly enhanced phagocytosis, presumably by allowing CR3 to become functional. Despite the likelihood that C3b as well as iC3b is deposited on *L. monocytogenes* during opsonization and the fact that CR1 can mediate low-affinity binding of iC3b, the divalent cation requirement suggests that CR1 is not a significant mediator of the phagocytosis of *L. monocytogenes* by inflammatory macrophages (37, 47). This conclusion is supported by the observation that the specific CR3 blockade by M1/70 was as effective as anti-C3 antibody or heat-inactivated serum in inhibiting phagocytosis.

CRs do not constitutively phagocytose ligand-bound targets; they need to be activated to do so (45, 60). Indeed, using mouse resident peritoneal macrophages, Noel et al. (38) found that although most *Haemophilus influenza* type b cells were bound to macrophage CR3 when incubated in nonimmune serum, only 11% were internalized. In the experiments reported here with inflammatory peritoneal macrophages, >95% of cell-associated bacteria were actually internalized by the phagocytes (Table 1). This high percentage of internalization was essentially the same for both live and heat-killed organisms and was not significantly different when bacteria were incubated in normal serum, heat-inactivated serum, no serum, or the presence of anti-CR3 antibody.

The high percentage of ingestion could be a reflection of the cell, the bacterium, or both. It is likely that CRs on phagocytes elicited by an inflammatory agent, such as proteose peptone, are already stimulated and that the cells are in a highly phagocytic state. Thioglycolate-elicited and glycogen-elicited mouse peritoneal macrophages have shown constitutive phagocytosis of E(IgM)C3b by CRs, whereas PECs elicited with other agents, such as live bacteria, have not (6, 19, 32, 49). On the other hand, *L. monocytogenes* may have surface properties that favor its internalization once it is bound to a cell. For instance, a 60-kDa surface protein which is essential for *L. monocytogenes* to invade the mouse fibroblast cell line 3T6 has been described (26). The interaction of this protein with professional phagocytes, however, has not been explored. It seems that the most likely explanation for the high percentage of internalization is that the cells used in our assay, macrophages recruited to an inflammatory focus, are by nature highly phagocytic cells with activated CRs.

Facultative intracellular bacteria have been divided into separate groups based on their intracellular behavior (35). One group includes *Mycobacterium*, *Salmonella*, *Yersinia*, and *Legionella* spp., which reside and multiply within phagocytic vacuoles in the host cell. The other group includes bacteria which escape the phagosome and replicate freely in the host cell cytoplasm. This second group consists of *Listeria* and *Shigella* spp., both of which utilize a hemolysin to lyse the endosome soon after entering the host cell (43, 51). Other similarities between these two bacteria are their ability to invade nonprofessional phagocytes and their dependence upon host cell F-actin to move intracellularly and infect adjacent cells (5, 10, 36, 56).

The mechanism by which facultative intracellular bacteria enter host cells has been studied in both professional and nonprofessional phagocytes. With regard to professional phagocytes, receptor-mediated entry has been documented for *M. tuberculosis*, *M. leprae*, *L. pneumophila*, and *L. monocytogenes* (42, 52, 53; this report). In each case, CR1, CR3, or both play prominent roles in the entry of these pathogens into host mononuclear phagocytes in the presence of serum. Of the bacteria studied above, only *L. monocytogenes* escapes the phagocytic vacuole. The other organisms have developed mechanisms which allow them to survive and multiply in the phagosome (16, 35). In nonprofessional phagocytes, *Yersinia pseudotuberculosis* employs an invasin protein which binds to β_1 integrin molecules on HEp-2 cells and the Re mutant of *S. minnesota* may use the C1q receptor to enter pulmonary endothelial cells (23, 50). Following phagocytosis, both of these bacteria, as well as other members of their genera, remain within phagosomes in the host cell (16, 35).

Several questions remain regarding the mode of entry of *L. monocytogenes* into inflammatory phagocytes. First, by what mechanism does the small amount of serum- and CR3-iC3b-independent phagocytosis of this bacterium occur? Several alternative means of entry exist. Phagocytosis in the absence of serum suggests a direct cell-bacterium interaction, for instance, by lectin-ligand interactions, in which phagocyte carbohydrate-binding proteins mediate binding and phagocytosis (40). One such example is the mannose-fucose receptor, which is present on many types of macrophages and can mediate the binding and phagocytosis of such diverse organisms as *Leishmania donovani* and *Escherichia coli* by mouse peritoneal macrophages (3, 55, 59). Furthermore, a lectin binding site on CR3 has been identified and may play a role in the serum-independent phagocytosis of *L. monocytogenes* (46).

Second, does the mode of entry of *L. monocytogenes* into phagocytes influence its intracellular fate? For instance, does phagocytosis by a given receptor lead to intracellular killing, whereas entry by a different means favors survival of the pathogen? Peptone-elicited peritoneal macrophages have been shown to be a model of listericidal mononuclear phagocytes (11, 54). In such cells, there may be a correlation between the major route by which *L. monocytogenes* is phagocytosed, in this case, C3-CR3-mediated interactions, and the subsequent intracellular death of this bacterium. Experiments designed to test this hypothesis are currently under way.

Certain lines of evidence from other experimental systems also suggest that ligand-receptor interactions may influence intracellular events. Joiner et al. (25) showed that following phagocytosis of *S. typhimurium* opsonized with either C3 or IgG, azurophil granule components were present in the phagosome in human neutrophils. In contrast, secondary granule constituents were incorporated into the phagosome following the phagocytosis of IgG-opsonized bacteria only. Additionally, Rothlein and Springer (49) demonstrated that CR3-mediated phagocytosis by thioglycolate-elicited peritoneal macrophages of erythrocytes opsonized with IgM and complement led to rapid intracellular degradation. This degradation could be blocked by M1/70 and contrasted with the fate of unopsonized erythrocytes, which were much less efficiently lysed following phagocytosis. Undoubtedly, the ultimate fate of a facultative intracellular bacterium after entry into a cell is the result of a complex series of interactions between the pathogen and the host, interactions which may be influenced by the receptor(s) mediating phagocytosis.

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