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Complement Receptor Type 3 (CD11b/CD18) Involvement is Essential for Killing of *Listeria monocytogenes* by Mouse Macrophages[1]

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Abstract. Recent work indicated that C receptor type 3 (CR3) mediates most phagocytosis of the facultative intracellular bacterium *Listeria monocytogenes* by mouse macrophages, which can kill it. In contrast, phagocytosis of *Listeria* by a population of nonlistericidal macrophages was largely CR3-independent. These findings suggested that CR3 binding during phagocytosis may be important in determining whether a macrophage kills *Listeria*, or is parasitized by the bacterium. The experiments reported here tested this hypothesis. When phagocytosis and killing were assayed separately, normally listericidal peritoneal macrophages still could phagocytose to some extent, but lost listericidal activity when CR3 was blocked by mAb. Anti-CR3 mAb inhibited killing in a dose-dependent fashion, and at high doses the cells became permissive hosts. Microbicidal function also was inhibited when active C components were absent during phagocytosis and during killing. Because *Listeria* are confined to phagosomes in listericidal macrophages but escape into the cytoplasm in nonlistericidal macrophages, we tested whether anti-CR3 mAb enhanced phagosomal escape. In fact, escape of *Listeria* into the cytoplasm was rare in both control and anti-CR3 mAb-treated macrophages. Moreover, electron microscopy of these cells demonstrated dividing intraphagosomal bacteria. Taken together, these results suggest that binding to CR3 during phagocytosis leads to bacterial killing, and that phagocytic pathways engaged when binding to CR3 is blocked do not trigger microbicidal activity. Furthermore, restriction of *Listeria* to the phagosome in the absence of CR3 engagement is not by itself sufficient for macrophage listericidal activity. *Journal of Immunology,* 1993, 151: 5431.

Facultative intracellular bacteria are pathogens characterized by their ability to enter and replicate within eukaryotic cells (1–3). Entry into host cells is accomplished by one of two general mechanisms: invasion of nonphagocytic cells, or phagocytosis by professional phagocytes (1–5). The former is termed "parasite-directed endocytosis," in which bacteria bind the target cell, then induce their own internalization. Once inside a nonprofessional phagocyte, the bacteria usually encounter an environment in which they proliferate unchecked. In contrast, phagocytosis by professional phagocytes is conceptually different from at least two standpoints. 1) Internalization of bacteria by phagocytes typically is a "host-directed" event initiated when phagocytic receptors bind specific ligands on the bacteria. 2) Perhaps more importantly, professional phagocytes may be armed with potent microbicidal mechanisms that intracellular bacteria must alter or avoid to survive (6, 7).

Many studies of macrophage interactions with facultative intracellular bacteria have centered on how these pathogens frustrate the host's microbicidal capabilities. Recently, the means by which one such organism, *Listeria monocytogenes*, parasitizes macrophages has been de-

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scribed. After phagocytosis by permissive cells, *Listeria* readily lyse the phagosome and then replicate in the cytoplasm (8, 9). In contrast, listericidal macrophages prevent phagosomal escape and are able to kill the bacterium (10, 11).

Little is known about host factors that lead to macrophage killing of facultative intracellular bacteria. In this capacity, some investigations have focused on how mononuclear phagocytes bind and phagocytose these pathogens. They show that the C receptors type 1 (CD35), type 3 (CR3,[3] CD11b/CD18), and type 4 (CD11c/CD18) mediate phagocytosis of intracellular bacteria such as *Legionella pneumophila*, *L. monocytogenes*, *Mycobacterium avium-intracellulare*, *Mycobacterium leprae*, and *Mycobacterium tuberculosis*, and certain *Salmonella* species (12–18). These results are significant because a growing body of evidence suggests that receptor: ligand interactions between host cells and pathogens during phagocytosis may influence or perhaps direct the post-phagocytic fate of intracellular parasites (3, 19, 20).

To address this complex and important issue, we studied phagocytosis of the facultative intracellular bacterium *L. monocytogenes* by different populations of mouse peritoneal macrophages. These cells are heterogeneous regarding their ability to kill *Listeria*, and may be used to identify host characteristics that lead to bacterial killing or permissive intracellular growth. Listericidal macrophages use CR3 as the major phagocytic receptor to bind and internalize *Listeria* (13). In contrast, CR3 mediates only a small amount of phagocytosis by a population of nonlistericidal macrophages (11). Therefore, we hypothesized that CR3-mediated phagocytosis of *Listeria* is required for macrophages to kill this bacterium, and that phagocytosis through other receptor(s) leads to permissive growth. The experiments reported here confirm this hypothesis.

Materials and Methods

Mice

(C57Bl/6 × DBA/2) F_1 (hereafter referred to as BDF_1) mice were obtained from The Jackson Laboratory, Bar Harbor, ME. Animals were housed in the barrier quarantined, specific pathogen free National Jewish Center for Immunology and Respiratory Medicine Animal Care Facility, fed mouse chow, and given water ad libitum. Female mice were used at 8 to 16 wk of age.

Bacteria

L. monocytogenes, strain EGD, was maintained in a virulent state by periodic passage in BDF_1 mice, and was stored at −70°C in 0.5-ml aliquots of a log phase culture. For each experiment, an aliquot of bacteria was thawed, and approximately 30 μl inoculated into 5 ml tryptose phosphate broth (Difco, Detroit, MI), and then incubated overnight at 37°C with constant agitation. The bacteria were pelleted and washed with BSS (21), and the bacterial concentration was determined by counting in a Petroff-Hausser chamber.

Sera

NMS was obtained from C57Bl/6 retired breeders (The Jackson Laboratory). The mice were exsanguinated by cardiac puncture under Metofane (Pitman-Moore, Inc., Washington Crossing, NJ) anesthesia, and serum was separated and stored at −70°C. An aliquot was thawed immediately before being used in each experiment. HIMS was prepared by heating normal serum to 56°C for 30 min, and C depletion was verified as described (13).

Antibodies

The anti-CR3 mAb, M1/70, is a rat IgG2b specific for mouse and human CR3 and blocks binding of iC3b-coated targets to CR3 (22). Antibody was purified from hybridoma supernatant and $F(ab')_2$ fragments were obtained as described previously (13). BM8, a rat IgG2a directed against a 125-kDa glycoprotein on mouse mononuclear phagocytes, was obtained from BMA Biomedicals AG, Augst, Switzerland (23). Affinity-purified phycoerythrin-labeled $F(ab')_2$ fragments of donkey anti-rat IgG (Jackson Immunoresearch Laboratories, West Grove, PA) were used as second step conjugates in flow-cytometric double labeling experiments. Control experiments showed that antibodies used in the bactericidal assay had no significant inhibitory or stimulatory effect upon bacterial growth in cell-free suspensions.

Cells

PEC were obtained by injecting mice i. p. with 1.0 ml sterile 10% proteose peptone (Difco, Detroit, MI). Cells were harvested 48 h later by peritoneal lavage with 10 ml cold, sterile BSS. The cells were collected by centrifugation at 250 × *g* for 10 min, and then resuspended in BSS and counted in a hemocytometer.

Binding and internalization of opsonized *Listeria* by PEC

Listeria were incubated in BSS with 5% NMS or HIMS as a source of opsonin for 30 min at 37°C, then cooled on ice for 15 min. To block CR3-mediated phagocytosis of NMS-opsonized *Listeria*, aliquots of cells were incubated for 30 to 45 min on ice with $F(ab')_2$ fragments of anti-CR3 mAb, or control antibody where indicated. To avoid early killing

[3] Abbreviations used in this paper: CR3, C receptor type 3; BSS, balanced salt solution; NMS, normal mouse serum; HIMS, heat-inactivated mouse serum; PEC, peritoneal exudate cells.

of bacteria as reported by others (24, 25), opsonized bacteria were mixed with PEC in the cold (approximately 7°C) for 20 min. This procedure allowed cells to bind, but not internalize bacteria (26) (see below), and therefore prevented intracellular killing as well. Unbound bacteria were removed by washing the cells twice with iced BSS followed by centrifugation through a 1-ml layer of 30% sucrose for 10 min at 4°C (27).

To verify that bacteria bound to PEC in the cold were extracellular, we used fluorescence microscopy to discriminate between intracellular and extracellular bacteria as described previously (28). Briefly, FITC-labeled, heat-killed *Listeria* were incubated in 5% NMS or HIMS for 30 min at 37°C, iced, then mixed with PEC with or without $F(ab')_2$ fragments of anti-CR3 mAb as described above. After washing, samples from each group were kept on ice, or warmed in a 37°C water bath for 15 min. Ethidium bromide was added to a final concentration of 50 μg/ml, and the cells were observed by fluorescence microscopy to determine the numbers of intracellular (green fluorescent) and extracellular (red-orange fluorescent) bacteria before and after warming. At time zero, greater than 85% of cell-associated *Listeria* were extracellular, but internalization proceeded rapidly upon warming. After 15 min at 37°C, greater than 85% of bound bacteria were intracellular in each experimental group. Other experiments using confocal microscopy showed that 100% of cell-associated bacteria were intracellular after 30 min of incubation.

To test whether inhibition of phagocytosis by anti-CR3 mAb was measured accurately by CFU of PEC-associated *Listeria*, PEC and NMS-opsonized bacteria were mixed with or without 3.0 μg/ml $F(ab')_2$ fragments of anti-CR3 mAb as described above. The cells were washed and aliquots were removed, serially diluted in distilled water, and then plated on tryptic soy agar (Remel, Lenexa, KS), and CFU calculated. The remaining cells were warmed to 37°C for at least 15 min, and then cytocentrifuge preparations were made and stained with Diff-Quik (Baxter Healthcare Corp., McGaw Park, IL) to measure phagocytosis by light microscopy. Stained preparations were examined microscopically under oil immersion (1000×) by counting 150 to 200 cells/slide. Results were expressed as Phagocytic Index, calculated as (percent macrophages containing ≥ 1 bacterium) × (mean bacteria per positive cell) as described previously (13, 29).

Intracellular killing assay

To test the ability of PEC to kill *Listeria*, a modification of previously described microbicidal assays was performed in the complete absence of antibiotics (24, 29). *Listeria* were opsonized in NMS or HIMS as described above, and then were mixed 1:1 with PEC in the presence or absence of $F(ab')_2$ fragments of anti-CR3 mAb, or control antibody as indicated. After washing, cells with bound bacteria were resuspended in BSS, and then 100-μl aliquots were removed for serial dilution in sterile distilled water and plating to determine the number of cell-associated bacteria at time zero. The remaining cells were warmed to 37°C for 15 min to allow internalization of bound bacteria, and then were aliquoted into tubes containing 5% serum with or without anti-CR3 antibody. The tubes then were incubated for 90 min at 37°C to allow killing or growth of *Listeria*. After this time, aliquots containing the same number of PEC as plated at time zero were lysed and plated to determine CFU remaining. Bacterial killing was detected as a decrease in CFU from time zero to time 105, whereas bacterial growth caused an increase in CFU over the same time period. Control experiments showed that cell viability determined by trypan blue exclusion was >95% after the 90-min bacterial killing/growth period in both anti-CR3-treated and untreated groups.

Identification of phagocytic subpopulations in PEC

PEC were incubated with heat-killed FITC-labeled *Listeria* at a 1:1 ratio in the presence of 5% NMS or 5% NMS with 1 μg/ml (final concentration) $F(ab')_2$ fragments of anti-CR3 mAb for 30 min at 37°C under continuous rotation. The cells were centrifuged and washed with BSS, and subsequently labeled for flow cytometry using M1/70 hybridoma supernatant or negative control supernatant, followed by optimally diluted phycoerythrin-labeled anti-rat conjugate. After each labeling step, the cells were washed three times with PBS containing 2% BSA and 0.2% azide. Phagocytosis of FITC-*Listeria* and binding of M1/70 mAb by PEC subpopulations were assessed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). E and dead cells were excluded from analysis by gating on the basis of forward and side scatter characteristics.

Assay for cytoplasmic location of *Listeria*

The proportion of phagocytosed *Listeria* that escape the phagosome and become coated with F-actin was measured as described previously (11). Macrophages were incubated on ice for 30 min with or without 3.0 μg/ml anti-CR3 mAb. Next, BSS with 5% NMS, and *Listeria* at 1:4 cell:bacteria ratio, were added simultaneously and the mixture of cells and bacteria was incubated for 30 min at 37°C. Cells were washed to remove unattached bacteria; 100-μl aliquots were removed, cytocentrifuged, and then stained with Diff-Quik; and the phagocytic index determined. The remaining cells were incubated for 120 min at 37°C and then cytocentrifuged onto glass slides, fixed with 3.7% formaldehyde, quenched with 0.1 M glycine, and permeabilized with 0.2% Triton X-100 (Sigma Chemical Co., St. Louis, MO) (30). Actin-coated *Listeria* were imaged by staining cells with 10 U/ml Bodipy 581/591 phallicidin (Molecular

Probes, Eugene, OR) for 20 min. Cells were examined using a Leitz Diaplan microscope with epifluorescence under oil immersion (1000×) with a rhodamine filter set. To quantify actin-coated bacteria, an actin index was determined in the same fashion as for phagocytosis. That is, 200 to 300 cells/slide were counted, and actin index was calculated as: (% cells containing ≥ 1 actin-coated bacterium) × (mean actin-coated bacteria/positive cell) (11).

Electron microscopy of *Listeria*-infected peritoneal cells

To identify more precisely the intracellular compartment of replicating *Listeria*, transmission electron microscopy of *Listeria*-infected PEC was performed as described previously (11). Briefly, PEC and *Listeria* were mixed 1:2 in the absence, or 1:6 in the presence of 1 μg/ml anti-CR3 mAb, in BSS with 5% NMS for 30 min at 37°C. The cells were washed and then incubated for 2 or 3 h in 5% NMS before fixation with 3% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.3. One percent tannic acid and 1.5% glutaraldehyde in 0.1 M cacodylate buffer were used as a secondary fixative. Cells were postfixed in 1% osmium tetroxide in 0.1 M cacodylate, dehydrated through a graded series of acetones, and infiltrated into Luft's 3:7 embedding resin. Uranyl acetate and Reynold's lead-stained sections were evaluated and photographed using a Phillips 400T electron microscope at an accelerating voltage of 60 kV.

Results

CR3 mediates binding and phagocytosis of *Listeria*

To test the effect of CR3-blockade on killing, we modified previously described killing assays to measure binding and killing separately. Therefore, initial experiments were performed to test whether the proportion of *Listeria* bound and internalized through CR3 in this system was measured accurately by counting CFU of PEC-associated *Listeria*. Opsonized *Listeria* were mixed 10:1 with PEC in the cold for 20 min, and then unbound bacteria were removed by washing. The cells were warmed to 37°C for 10 min, and uptake of bacteria was measured both by determining CFU of cell-associated bacteria and by light microscopy of cytocentrifuge preparations of PEC. When measured by microscopy, 3.0 μg/ml anti-CR3 antibody decreased the phagocytic index from 342 ± 65 (mean ± SEM) in control PEC to 111 ± 24 in mAb treated cells, a 65.8% reduction (n = 3). Under the same conditions, CFU of cell-associated bacteria decreased from 5.91 ± 0.04 $\log_{10}$ bacteria/ml in control cells to 5.38 ± 0.13 $\log_{10}$ bacteria/ml in the anti-CR3-treated group, a reduction of 70.5%. When opsonized *Listeria* and PEC were mixed in a 1:1 cell:bacteria ratio in the bactericidal assay, there was a 77% inhibition of bacterial uptake (see below). Therefore, under the conditions used in the bactericidal assay, CR3 mediated approximately 70% of binding and subsequent uptake of opsonized *Listeria* by PEC, similar to previous results (11, 13). Moreover, the amount of inhibition was similar whether measured by microscopy or CFU.

Table I
Anti-CR3 mAb inhibits listericidal activity of PEC

Binding Conditions[a]	Conditions during the Killing/Growth Period[b]	Bacterial Growth[c]
NMS	NMS	−0.36 ± 0.02[d]
	HIMS	−0.36 ± 0.07
	NMS + anti-CR3 mAb	−0.11 ± 0.02[d]
HIMS	NMS	−0.25 ± 0.08
	HIMS	+0.06 ± 0.03
	NMS + anti-CR3 mAb	−0.02 ± 0.11
NMS + anti-CR3 mAb[e]	NMS	+0.22 ± 0.08
	HIMS	+0.34 ± 0.04
	NMS + anti-CR3 mAb	+0.31 ± 0.02

[a] *Listeria* were opsonized in 5% serum as indicated then mixed 1:1 with PEC for 20 min in the cold.

[b] Cells were washed to remove unbound bacteria, time zero, then were warmed for 15 min to allow internalization. Next, aliquots of cells were mixed with NMS, HIMS, or NMS plus 3.0 μg/ml $F(ab')_2$ fragments of anti-CR3 mAb and incubated for another 90 (time 105) min to allow bacterial killing or growth.

[c] Calculated as the $\Delta\log_{10}$ CFU *Listeria* from time zero to time 105. A reduction of CFU denotes bacterial killing, whereas an increase indicates bacterial growth. Results from three experiments are presented as the mean ± SEM $\log_{10}$ CFU *Listeria*.

[d] The difference between these groups is statistically significant at p = 0.007 by Student's t test.

[e] PEC were incubated with 3.0 μg/ml $F(ab')_2$ fragments of anti-CR3 mAb before adding *Listeria*.

Effect of CR3 blockade on intracellular killing of *Listeria*

Next, we tested whether inhibition of CR3-mediated phagocytosis altered the ability of PEC to kill intracellular *Listeria*. NMS-opsonized *Listeria* were killed regardless of conditions during the killing/growth period, but killing was decreased when anti-CR3 mAb was present (Table I). *Listeria* opsonized in HIMS were killed only when NMS was added during the killing/growth period. When either HIMS or NMS with anti-CR3 mAb was used, killing was completely inhibited. When PEC were treated with anti-CR3 mAb before binding NMS-opsonized *Listeria*, there was significant bacterial growth regardless of later incubation conditions. This effect of anti-CR3 mAb on killing was dose-dependent and paralleled inhibition of phagocytosis (Fig. 1). These data suggest that CR3-mediated phagocytosis of *Listeria* leads to bactericidal activity. When CR3-mediated phagocytosis was prevented by mAb, or by completely excluding C from the system, the PEC lost microbicidal ability. Taken together, these data indicate that inhibition of binding of iC3b to CR3, even if only during the killing/growth period, inhibits killing of *Listeria* by PEC.

To test the specificity of CR3 blockade on uptake and

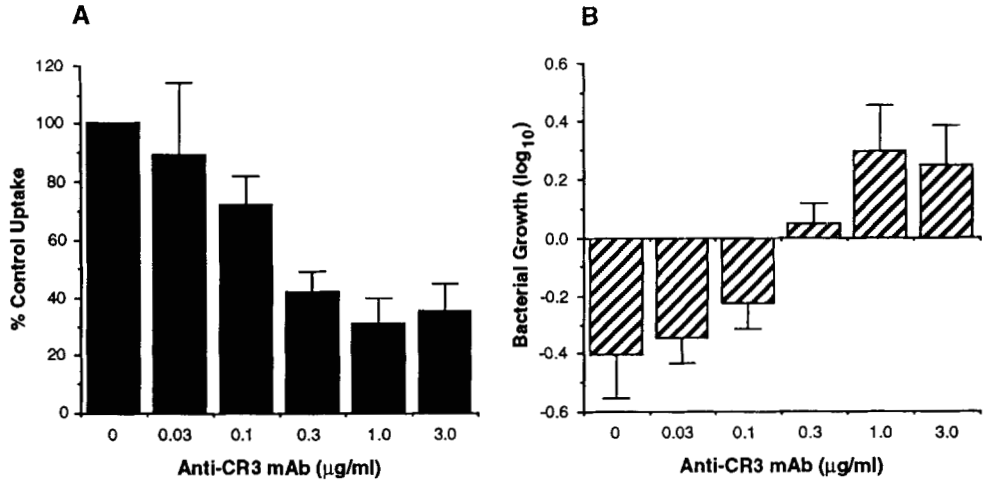

FIGURE 1. Anti-CR3 mAb causes a dose-dependent inhibition of uptake and blocks killing of *Listeria*. PEC were treated with increasing amounts of anti-CR3 mAb, and then mixed for 20 min in the cold with *Listeria* opsonized in normal serum. The cells were washed and CFU of cell-associated bacteria were counted before (time zero) and after (time 105) a 105-min incubation to determine bacterial killing or growth. *A*, Bacterial uptake as measured by CFU of cell-bound bacteria at time zero. *B*, Killing or growth of *Listeria* calculated as $\log_{10}$ bacteria (time zero) – (time 105). Results are presented as the mean ± SEM of four or five experiments.

killing, BM8, a rat IgG2a that binds mouse macrophages, was used at 1.0 μg/ml, and its effect compared to cells without antibody and cells treated with 1.0 μg/ml $F(ab')_2$ fragments of anti-CR3 mAb as used in the killing assay. In these experiments, the mean ± SEM uptake measured by $\log_{10}$ CFU at time zero in the absence of mAb was 5.50 ± 0.06. This was decreased to 4.98 ± 0.5 with anti-CR3 mAb, but not with control mAb BM8, 5.45 ± 0.14 (n = 3). The change in CFU bacteria in control cells was −0.28 ± 0.10 log indicating killing, and was reversed in anti-CR3-treated cells to 0.26 ± 0.08 log growth. Again BM8 had no effect with a change in CFU of −0.22 ± 0.04 log (n = 4). These results demonstrate that inhibition of uptake and killing most likely was due to prevention of CR3 usage.

Identification of the phagocytic subpopulation of PEC

An alternative explanation for the observed blockade of bacterial killing by anti-CR3 mAb might be that, because a peritoneal exudate contains multiple different subpopulations of cells, anti-CR3 mAb may have prevented phagocytosis by a bactericidal subpopulation, whereas the residual phagocytic cells were not bactericidal. To identify the phagocytic PEC subpopulations in the presence and absence of anti-CR3 mAb, we let PEC phagocytose heat-killed FITC-labeled *Listeria* at the same 1:1 ratio used in the killing assay, and subsequently stained the cells for Mac-1 Ag expression (Fig. 2). Mac-1 expression was not increased upon exposure of cells to bacteria (data not shown). Whether or not anti-CR3 mAb was used to inhibit phagocytosis, the vast majority of phagocytic cells was found in the subpopulation with the highest level of Mac-1 expression. Actual isolation of these cells by cell sorting showed that they were predominantly monocytes and macrophages (data not shown). Furthermore, the scatter profiles of phagocytic subpopulations were identical under both conditions, showing low to intermediate forward scatter and intermediate to high side scatter. Therefore, these data strongly suggest that the same monocyte/macrophage PEC subpopulation phagocytoses opsonized *Listeria* in either the presence or absence of anti-CR3 mAb.

Intracellular location of *Listeria*

To provide an explanation for the loss of bactericidal activity, we tested whether anti-CR3 mAb-treated PEC were less able to prevent phagosomal escape of *Listeria*. PEC were incubated with NMS-opsonized *Listeria* with and without $F(ab')_2$ fragments of anti-CR3 mAb, then both phagocytosis and F-actin-coating as a marker for cytoplasmic location of *Listeria* were measured. In these experiments, anti-CR3 mAb decreased phagocytosis by 62% (Table II), but 2 h after phagocytosis, F-actin-coated bacteria were not found more frequently in anti-CR3 mAb-treated PEC than in control cells. These experiments demonstrated that phagosomal escape was not responsible for growth in anti-CR3-treated macrophages. They also raised the possibility that under these particular conditions, intracellular *Listeria* could replicate in phagosomes.

To confirm the results of fluorescence microscopy and to locate more precisely the intracellular compartment of replicating *Listeria* within anti-CR3-treated cells, electron microscopy of *Listeria*-infected cells was performed. PEC were infected with *Listeria* in the presence or absence of anti-CR3 mAb, washed, and then incubated for 2 and 3 h before fixation. In none of the cells examined were *Listeria*

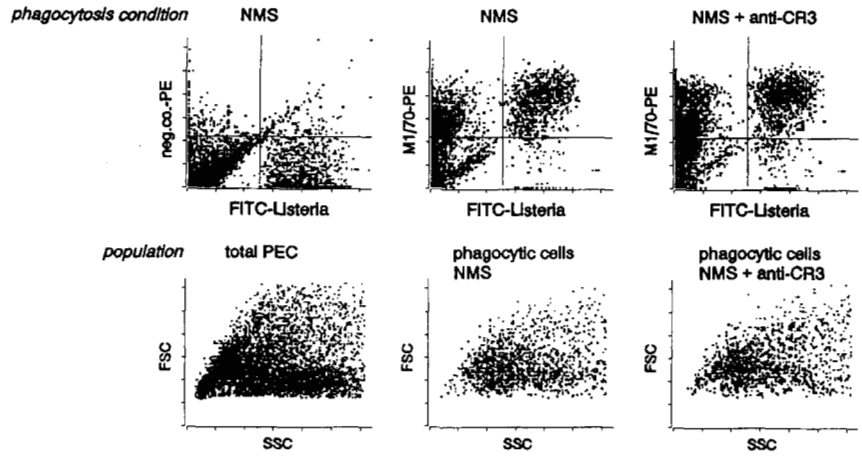

FIGURE 2. Same PEC subpopulation phagocytoses *Listeria*, in either the presence or absence of anti-CR3. PEC with or without incubation with anti-CR3 mAb (1 μg/ml final concentration), were allowed to phagocytose NMS-opsonized FITC-labeled heat-killed *Listeria*. Next, cells were labeled with M1/70 and anti-rat phycoerythrin, and fluorescence and scatter characteristics were determined by flow cytometry. To assess the scatter profiles of the phagocytic cells, the latter were selected by electronic gating on the basis of FITC fluorescence. FSC, forward scatter; SSC, side scatter.

Table II
Anti-CR3 mAb inhibits phagocytosis of Listeria but does not alter subsequent phagosomal escape

Cells[a]	Phagocytic Index[b]	Actin Index[c]	Escape Ratio[d]
Control	209 ± 24[e]	3.9 ± 1.4[e]	0.018 ± 0.005[e]
Anti-CR3-treated[f]	80 ± 7	1.2 ± 0.4	0.016 ± 0.004

[a] PEC were mixed 1:4 with *Listeria* plus 5% normal serum for 30 min at 37°C, then were washed. Aliquots of cells were removed to measure phagocytosis, and the remaining cells were incubated for 2 h with 5% normal serum.
[b] Cytocentrifuge preparations of cells were stained with Diff-Quik and examined by light microscopy. Phagocytic index = (% cells with ≥ 1 bacterium) × (mean bacteria/positive cell).
[c] Cytocentrifuge preparations were stained with Bodipy 581/591 phallicidin to label F-actin, and visualized by fluorescence microscopy. Actin index is calculated as (% cells with ≥ 1 F-actin-coated bacterium) × (mean bacteria/positive cell).
[d] Calculated as actin index/phagocytic index.
[e] Results are presented as mean ± SEM of six experiments.
[f] Cells were incubated with 3.0 μg/ml anti-CR3 mAb before addition of *Listeria*.

found externally bound to the cell or in the process of being phagocytosed, strengthening the notion that bacterial growth did not occur extracellularly. *Listeria* were easily identified within phagosomes in both control (data not shown) and anti-CR3-treated cells (Fig. 3). When the 2- and 3-h time points were compared, there was an obvious increase in the number of bacteria per infected cell in anti-CR3-treated PEC but not in control cells. Furthermore, after 3 h of incubation, many bacteria in the anti-CR3-treated cells were dividing within phagosomes (Fig. 3*a*), and several cells had more than one bacterium per phagosome, suggesting completed division (Fig. 3*b*). These findings were only rarely noted in control cells. These results support the fluorescence microscopy data indicating that phagocytosis was complete and that bacteria did not escape from the phagosomes of anti-CR3 mAb-treated cells. They also establish the phagosome as the site of intracellular replication in anti-CR3 mAb-treated PEC. Thus, both containment within the phagosome and iC3b-CR3 interaction are required for listericidal activity.

Discussion

Previous work from this laboratory showed that listericidal proteose peptone-elicited peritoneal cells phagocytosed *Listeria* mainly through CR3 (11, 13). This event was promoted by deposition of C3 split products, presumably iC3b, on the bacterial cell wall by the alternative C pathway (13). In contrast, phagocytosis of *Listeria* by thioglycollate-elicited peritoneal cells, a population that cannot kill *Listeria*, was mostly CR3-independent (11, 31, 32). On the basis of these observations, we hypothesized that CR3-mediated phagocytosis of *Listeria* results in microbial killing, whereas phagocytosis through a different receptor(s) leads to permissive intracellular growth. This is possible because certain post-phagocytic events important for microbicidal activity are initiated by specific receptor ligation. Examples of such events that are influenced by the opsonizing ligands and phagocytic receptors used include fusion of phagosomes with lysosomes, and with neutrophil granules (18, 19, 33). The purpose of the experiments presented here was to test the role of CR3 in macrophage killing of *Listeria*.

Initial experiments studied binding and internalization of bacteria by PEC. Under the conditions used to test for CR3-mediated killing, we confirmed the experimental separation of binding from internalization, and the contribution of CR3 to phagocytosis. Additional studies using conventional fluorescence microscopy, confocal scanning fluorescence microscopy, and electron microscopy each showed that despite overall reduction in quantity of phagocytosis,

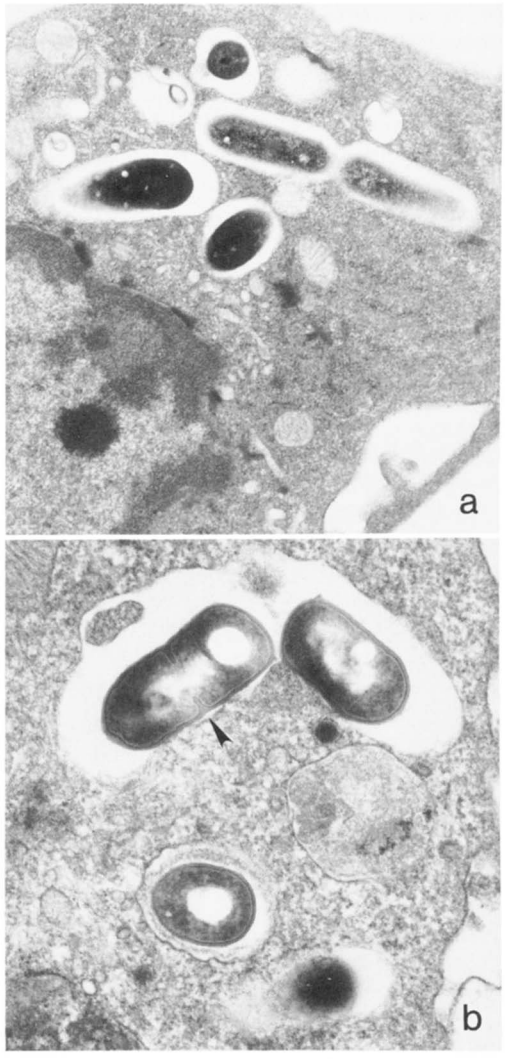

FIGURE 3. *Listeria* divide within phagosomes of anti-CR3 mAb-treated PEC. PEC with or without anti-CR3 mAb were allowed to phagocytose opsonized *Listeria* for 30 min, and then were washed and incubated another 3 h before fixation. *a* and *b* were taken from different anti-CR3 mAb-treated cells and were representative of other cells observed. *a*, PEC with several intraphagosomal *Listeria* including one in the later stages of division (×18,500). *b*, PEC showing a phagosome containing two discrete bacteria suggesting completed division. One of the bacteria is in early cell division as well (*arrow*) (×30,500).

PEC could bind and internalize *Listeria* through CR3-independent means as well as through CR3. These results also indicate that extracellular bacteria were thoroughly removed from the killing assay, and any remaining organisms probably were readily phagocytosed. Next, using flow-cytometric markers, we identified macrophages as the predominant phagocyte in the peritoneal exudate responsible for uptake of *Listeria* in the presence or absence of anti-CR3 mAb. Therefore, we are confident that this assay accurately measured intracellular killing of *Listeria* by macrophages.

The intracellular fate of *Listeria* phagocytosed through CR3 was determined by testing whether peritoneal macrophages could kill *Listeria* when CR3-mediated phagocytosis was inhibited. We found that normally listericidal cells lost their ability to kill *Listeria* when CR3-mediated binding and phagocytosis were blocked. Additionally, blockade of killing by anti-CR3 mAb was dose-dependent over a wide concentration range, and closely paralleled inhibition of phagocytosis. In fact, at higher doses of anti-CR3 mAb, the PEC appeared to be permissive hosts for *Listeria* replication. Interestingly, anti-CR3 mAb added to PEC after they phagocytosed opsonized bacteria inhibited, but did not abrogate, killing. A similar finding was reported when anti-CR3 mAb was added to mouse neutrophils after binding *Proteus mirabilis* (34).

These findings suggest that two different receptor-mediated events may occur. One is CR3 engagement during phagocytosis of C-opsonized bacteria. In this situation, CR3 likely initiates signals that stimulate the cells' microbicidal mechanisms. The other event is utilization of other receptor(s) when CR3-mediated phagocytosis is prevented. These alternative receptors appear unable to initiate intracellular killing, and thus normally listericidal PEC support intracellular growth of *Listeria*.

Since the intracellular life cycle of *Listeria* is well described, we could ask at which stage CR3 blockade affected microbial survival. In permissive cells, *Listeria* escape the phagosome, nucleate F-actin, and replicate in the cytoplasm (8, 9), but listericidal cells prevent phagosomal escape (10, 11). Therefore, we used F-actin coating of cytoplasmic *Listeria* as a marker for phagosomal escape, and tested whether anti-CR3 mAb treatment of peritoneal macrophages altered the macrophage's ability to prevent it. Interestingly, we found the same low frequency of phagosomal escape in listericidal macrophages and in macrophages rendered nonlistericidal by CR3 blockade. This result was confirmed by electron microscopy of *Listeria*-infected macrophages, which showed *Listeria* confined to phagosomes in control and anti-CR3-mAb-treated macrophages. These studies indicated that even though *Listeria* did not enter the cytoplasm of anti-CR3-mAb treated macrophages, they were able to divide within phagosomes. Taken together, these data suggest that macrophage killing of *Listeria* may be separated into two steps. The first is preventing phagosomal escape, an event necessary but not sufficient for microbicidal activity. The second step, bacterial killing by currently unknown mechanisms, appears to be initiated through CR3.

The finding that CR3-mediated phagocytosis leads to macrophage killing of *Listeria* may not be true for other facultative intracellular bacteria. Studies with *Legionella pneumophila*, *M. avium* complex, *M. leprae*, and *M. tuberculosis* demonstrate the importance of C receptor-mediated phagocytosis (12, 14–17). However, they suggest

that this uptake mechanism leads to intracellular growth, not bacterial killing. This difference may be explained by two important differences between the peptone-elicited peritoneal macrophages used in the present study and the human mononuclear phagocytes used in the others. First, there is an obvious functional difference in that peptone-elicited peritoneal macrophages express constitutive listericidal activity. In contrast, the human phagocytes used in the above mentioned studies must be stimulated to exhibit microbicidal activity against *L. pneumophila* and the different Mycobacterial species (35–37). Second, C receptor-mediated phagocytosis may not be sufficient for microbial killing. Other characteristics such as low amounts of intracellular iron and low transferrin receptor levels also are important for killing of *Legionella* and *Listeria* (38, 39). Thus, any route of entry into a permissive host may lead to intracellular growth, and CR3-mediated phagocytosis may not be the only requirement for killing of facultative intracellular bacteria.

In conclusion, these experiments support the hypothesis that the fate of facultative intracellular bacteria within macrophages is influenced by the receptors that mediate phagocytosis. This is probably because certain intracellular post-phagocytic events important for microbicidal activity are linked to, or initiated through, ligation of specific phagocytic receptors. Finally, recent studies have shown that CR3-dependent myelomonocytic cell recruitment is essential for overcoming *Listeria* infection in mice (40). The results presented here suggest that in addition to phagocytosis, CR3 is needed to trigger killing of this facultative intracellular bacterium by inflammatory mononuclear phagocytes. Therefore, it appears that CR3 has at least three important roles in combating *Listeria* infection: recruitment, phagocytosis, and signaling microbicidal activity.

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References

1. Moulder, J. W. 1985. Comparative biology of intracellular parasitism. *Microbiol. Rev. 49:298.*
2. Finlay, B. B., and S. Falkow. 1989. Common themes in microbial pathogenicity. *Microbiol. Rev. 53:210.*
3. Falkow, S., R. R. Isberg, and D. A. Portnoy. 1992. The interaction of bacteria with mammalian cells. *Annu. Rev. Cell Biol. 8:333.*
4. Isberg, R. R. 1991. Discrimination between intracellular uptake and surface adhesion of bacterial pathogens. *Science 252:934.*
5. Falkow, S. 1991. Bacterial entry into eukaryotic cells. *Cell 65:1099.*
6. Horwitz, M. A. 1982. Phagocytosis of microorganisms. *Rev. Infect. Dis. 4:104.*
7. Quie, P. G. 1983. Perturbation of the normal mechanisms of intraleukocytic killing of bacteria. *J. Infect. Dis. 148:189.*
8. Tilney, L. G., and D. A. Portnoy. 1989. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes. J. Cell Biol. 109:1597.*
9. Mounier, J., A. Ryter, M. Coquis-Rondon, and P. Sansonetti. 1990. Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocyte-like cell line Caco-2. *Infect. Immun. 58:1048.*
10. Portnoy, D. A., R. D. Schreiber, P. Connelly, and L. G. Tilney. 1989. γ-Interferon limits access of *Listeria monocytogenes* to the macrophage cytoplasm. *J. Exp. Med. 170:2141.*
11. Drevets, D. A., B. P. Canono, and P. A. Campbell. 1992. Listericidal and nonlistericidal mouse macrophages differ in complement receptor type 3-mediated phagocytosis of *L. monocytogenes* and in preventing escape of the bacteria into the cytoplasm. *J. Leukocyte Biol. 52:70.*
12. Payne, N. R., and M. A. Horwitz. 1987. Phagocytosis of *Legionella pneumophila* is mediated by human monocyte complement receptors. *J. Exp. Med. 166:1377.*
13. Drevets, D. A., and P. A. Campbell. 1991. Roles of complement and complement receptor type 3 in phagocytosis of *Listeria monocytogenes* by inflammatory mouse peritoneal macrophages. *Infect. Immun. 59:2645.*
14. Bermudez, L. E., L. S. Young, and H. Enkel. 1991. Interaction of *Mycobacterium avium* complex with human macrophages: roles of membrane receptors and serum proteins. *Infect. Immun. 59:1697.*
15. Schlesinger, L. S., and M. A. Horwitz. 1990. Phagocytosis of leprosy bacilli is mediated by complement receptors CR1 and CR3 on human monocytes and complement component C3 in serum. *J. Clin. Invest. 85:1304.*
16. Schlesinger, L. S., and M. A. Horwitz. 1991. Phagocytosis of *Mycobacterium leprae* by human monocyte-derived macrophages is mediated by complement receptors CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) and IFN-γ activation inhibits complement receptor function and phagocytosis of this bacterium. *J. Immunol. 147:1983.*
17. Schlesinger, L. S., C. G. Bellinger-Kawahara, N. R. Payne, and M. A. Horwitz. 1990. Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. *J. Immunol. 144:2771.*
18. Ishibashi, Y., and T. Arai. 1990. Roles of the complement receptor type 1 (CR1) and type 3 (CR3) on phagocytosis and subsequent phagosome-lysosome fusion in *Salmonella*-infected murine macrophages. *FEMS Microbiol. Immunol. 64:89.*
19. Joiner, K. A., S. A. Fuhrman, H. M. Miettinen, L. H. Kasper, and I. Mellman. 1990. *Toxoplasma gondii:* Fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. *Science 249:641.*
20. Conlan, J. W., and R. J. North. 1992. Early pathogenesis of infection in the liver with the facultative intracellular bacteria *Listeria monocytogenes, Francisella tularensis,* and *Salmonella typhimurium* involves lysis of infected hepatocytes by leukocytes. *Infect. Immun. 60:5164.*
21. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med. 126:423.*
22. Beller, D. I., T. A. Springer, and R. D. Schreiber. 1982. Anti-mac-1 selectively inhibits the mouse and human type three complement receptor. *J. Exp. Med. 156:1000.*

23. Malorny, U., E. Michels, and C. Sorg. 1986. A monoclonal antibody against an antigen present on mouse macrophages and absent from monocytes. *Cell Tissue Res. 243:421.*
24. Leijh, P. C. J., M. T. van den Barselaar, T. L. van Zwet, M. R. Daha, and R. van Furth. 1979. Requirement of extracellular complement and immunoglobulin for intracellular killing of micro-organisms by human monocytes. *J. Clin. Invest. 63:772.*
25. Newman, S. L., and M. A. Tucci. 1990. Regulation of human monocyte/macrophage function by extracellular matrix. *J. Clin. Invest. 86:703.*
26. Lukacs, G. L., O. D. Rotstein, and S. Grinstein. 1990. Phagosomal acidification is mediated by a vacuolar-type H^+-ATPase in murine macrophages. *J. Biol. Chem. 265:21099.*
27. Weiss, J., L. Kao, M. Victor, and P. Elsbach. 1985. Oxygen-independent intracellular and oxygen-dependent extracellular killing of *Escherichia coli* S15 by human polymorphonuclear leukocytes. *J. Clin. Invest. 76:206.*
28. Drevets, D. A., and P. A. Campbell. 1991. Macrophage phagocytosis: use of fluorescence microscopy to distinguish between extracellular and intracellular bacteria. *J. Immunol. Methods 142:31.*
29. Czuprynski, C. J., P. M. Henson, and P. A. Campbell. 1984. Killing of *Listeria monocytogenes* by inflammatory neutrophils and mononuclear phagocytes from immune and non-immune mice. *J. Leuk. Biol. 35:193.*
30. Wang, K., J. R. Feramisco, and J. F. Ash. 1982. Fluorescent localization of contractile proteins in tissue culture cells. *Methods Enzymol. 85:514.*
31. Baker, L. A., and P. A. Campbell. 1980. Thioglycolate medium decreases resistance to bacterial infection in mice. *Infect. Immun. 27:455.*
32. Miake, S., K. Takeya, T. Matsumoto, Y. Yoshikai, and K. Nomoto. 1980. Relation between bactericidal and phagocytic activities of peritoneal macrophages induced by irritants. *J. Reticuloendothel. Soc. 4:421.*
33. Joiner, K. A., T. Ganz, J. Albert, and D. Rotrosen. 1989. The opsonizing ligand on *Salmonella typhimurium* influences incorporation of specific, but not azurophil, granule constituents into neutrophil phagosomes. *J. Cell Biol. 109:2771.*
34. Hart, P. H., L. K. Spencer, A. Nikoloutsopoulos, A. F. Lopez, M. A. Vadas, P. J. McDonald, and J. Finlay-Jones. 1986. Role of cell surface receptors in the regulation of intracellular killing of bacteria by murine peritoneal exudate neutrophils. *Infect. Immun. 52:245.*
35. Horwitz, M. A., and S. C. Silverstein. 1981. Activated human monocytes inhibit the intracellular multiplication of Legionnaires' disease bacteria. *J. Exp. Med. 154:1618.*
36. Bermudez, L. E. M., and L. S. Young. 1988. Tumor necrosis factor, alone or in combination with IL-2, but not IFN-γ, is associated with macrophage killing of *Mycobacterium avium* complex. *J. Immunol. 140:3006.*
37. Denis, M. 1991. Killing of *Mycobacterium tuberculosis* within human monocytes: activation by cytokines and calcitriol. *Clin. Exp. Immunol. 84:200.*
38. Byrd, T. F., and M. A. Horwitz. 1989. Interferon *gamma*-activated human monocytes downregulate transferrin receptors and inhibit the intracellular multiplication of *Legionella pneumophila* by limiting the availability of iron. *J. Clin. Invest. 83:1457.*
39. Alford, C. A., T. E. King, Jr., and P. A. Campbell. 1991. Role of transferrin, transferrin receptors, and iron in macrophage listericidal activity. *J. Exp. Med. 174:459.*
40. Rosen, H., S. Gordon, and R. J. North. 1989. Exacerbation of murine listeriosis by a monoclonal antibody specific for the type 3 complement receptor of myelomonocytic cells. *J. Exp. Med. 170:27.*