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Activation of the Human Complement Alternative Pathway by *Listeria monocytogenes*: Evidence for Direct Binding and Proteolysis of the C3 Component on Bacteria

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The capacity of the intracellular pathogen *Listeria monocytogenes* to activate the alternative pathway of human complement was examined. Incubation of *L. monocytogenes* with human serum in optimal conditions (20% Mg^{2+}EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]-chelated serum) consumed (31.3 ± 3.9)% of C3 hemolytic activity and led to similar amounts of C3 deposition among the 27 strains tested, except for a rough mutant and the penicillin-induced L forms of strain EGD, which bound reduced amounts of C3. The same results were obtained with strains belonging to related species (*L. innocua*, *L. seeligeri*, *L. welshimeri*, and *L. ivanovii*). Direct evidence is provided that *L. monocytogenes* induces the deposition of C3b and its cleavage products iC3b and C3d through ester and amide linkages, as demonstrated by the analysis of the released products of radiolabelled purified C3 after treatment with hydroxylamine. Our results clearly demonstrate that *L. monocytogenes* activates the alternative pathway of human complement, suggesting that bacteria in the blood or in tissues of infected patients are opsonized and targeted to C3 receptor-bearing cells such as macrophages.

Complement comprises a set of proteins that work to eliminate microorganisms from tissues and the blood. C3, the third complement protein, plays a central role in both the classical and the alternative (AP) pathways of complement activation. Upon C3 cleavage, opsonic C3b binds covalently through ester or amide bonds to nucleophilic acceptors of the activating surface via a transacylation reaction involving a newly exposed carbonyl group (23). Simultaneously, the activation of C3 results in the exposure on its α′ chain of a free -SH group, which may form disulfide bonds catalyzed by protein disulfide-isomerases such as thioredoxin (15). Invading microorganisms carrying C3b are marked for death either through a cytolytic action of the membrane attack complex of complement proteins C5-C9 or by phagocytosis by macrophages carrying CR1 receptors for bound C3b or CR3 receptors for bound iC3b (7). Alternatively, intracellular pathogens may utilize complement receptors to gain entry to host cells and, thereafter, use their environment as a haven from host defenses.

Listeria monocytogenes is a gram-positive facultative intracellular pathogen that can cause severe infections in humans and animals. The outcome of infection depends upon many properties of both host and bacteria, and some of the latter have recently been unraveled by transposon mutagenesis and gene cloning (10, 11, 19, 22, 25). Although protective immunity against this organism is mediated by *Listeria*-specific T cells and activated macrophages, serum factors including the complement system may play a role in the early stages of listeriosis. Components of the *Listeria* cell wall activate complement by the AP, thereby releasing chemotactic peptides for phagocytic cells (2) and possibly favoring their invasion via complement receptors for C3 fragments (9). However, the extent and the regulation of complement activation by various strains of *L. monocytogenes* and related species are poorly documented. Moreover, previous reports dealt with animal, and not human, serum as a source of complement.

The present experiments were designed to ascertain the ability of *L. monocytogenes* to activate the AP of complement in human serum. The resulting deposition and consumption of C3 among different strains of *L. monocytogenes* and related species, as well as the mode of C3 binding and the pattern of C3 fragments bound to bacteria, were studied.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Listeria* strains used in this study are listed in Table 1. These comprised 27 *L. monocytogenes* strains including 2 reference strains (R1 and -2), 8 mutants (M1 to -8) obtained, with the exception of SLCC5779, by transposon mutagenesis, 8 strains isolated from patients (P1 to -8), 9 strains isolated from food (F1 to -5) or animals (A1 to -4), and 4 strains belonging to the related species *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. ivanovii* (L1 to -4).

Bacteria were grown in brain heart infusion broth (Sanofi Diagnostics Pasteur) at 37°C with shaking, and log-phase cultures were stored at −70°C in 0.5-ml aliquots. The bacterial concentration was determined by plating bacteria at appropriate dilutions onto sheep blood agar (bioMérieux) plates and counting colonies the next day. Some mutant strains were grown in media supplemented with 5 mg of erythromycin or 20 mg of kanamycin per liter. For some experiments, bacteria were killed by heating at 65°C for 1 h or treated with the protease trypsin (0.5 mg/ml) or pronase (1 mg/ml) for 10 min at 37°C prior to use. Cell wall-deficient *Listeria* organisms (L forms) were produced by a method adapted from that of Brem and Eveland (6), based on a 3-h preculture of strain EGD in brain heart infusion-sucrose (0.5

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TABLE 1. *Listeria* strains tested

Sample	Strain[a]	Sero-var	Description or source of isolate	Reference or origin
R1	EGD	1/2a		11
R2	LO28	1/2c		28
M1	BUG5	1/2a	*inlA*[b]	10
M2	LUT12	1/2c	*actA*[b]	19
M3	CNL85/162	1/2a	*hly*[b]	11
M4	CNL85/163	1/2a	*hly*[+c]	11
M5	JL762	1/2a	*mpl*[b]	25
M6	BUG660	1/2c	*plcB*[b]	19
M7	BUG206	1/2c	*plcA*[b]	22
M8	SLCC5779	1/2a	Rough	14
P1	GR9201	1/2a	Human	Personal collection[d]
P2	GR9202	1/2b	Human	Personal collection
P3	GR9203	1/2c	Human	Personal collection
P4	GR9204	1/2c	Human	Personal collection
P5	GR9205	4b	Human	Personal collection
P6	GR9206	1/2a	Human	Personal collection
P7	GR9207	1/2b	Human	Personal collection
P8	GR9208	4b	Human	Personal collection
A1	GR9210	1/2a	Animal	Personal collection
A2	GR9211	1/2a	Animal	Personal collection
A3	GR9212	1/2a	Animal	Personal collection
A4	GR9213	4b	Animal	Personal collection
F1	GR9214	1/2a	Raw milk	Personal collection
F2	GR9215	1/2a	Cheese	Personal collection
F3	GR9216	1/2a	Cheese	Personal collection
F4	GR9217	1/2b	Cheese	Personal collection
F5	GR9218	1/2b	Cheese	Personal collection
L1	*L. innocua*	3	Human	Personal collection
L2	*L. welshimeri* SLCC5334	6		
L3	*L. seeligeri* SLCC3503	1/2a		
L4	*L. ivanovii* SLCC4121	5		

[a] *L. monocytogenes* unless specified otherwise. SLCC, Special *Listeria* Culture Collection; CNL, Center National *Listeria*.
[b] Mutant defective for the indicated gene.
[c] Revertant from the *hly* mutant.
[d] Personal collection, Microbiology Laboratory, Grenoble, France.

M) followed by the addition of 20 mg of penicillin G per liter for 4 h.

Complement source and protein C3. Normal human serum (NHS) was obtained from two healthy volunteers, pooled, and immediately frozen at −70°C. The search for anti-*L. monocytogenes* antibodies by agglutination and by a dot blot technique specific for anti-listeriolysin antibodies (3) was negative. This NHS pool was treated with methylamine, as described elsewhere (18), to inactivate C3 (and C4) through cleavage of its internal thioester bond. AP hemolytic activity (see below) was absent in methylamine-treated serum and was fully restored upon addition of purified human C3 at 0.5 mg/ml. Serum was also obtained from a patient with congenital C2 deficiency. This serum retained full AP hemolytic activity but exhibited <5% of the classical pathway hemolytic activity.

Purified C3 was prepared from pooled human plasma as described by Al Salihi et al. (1) and iodinated by the IODOGEN method. Specific activity of the labelled preparation was 2.5×10^7 cpm/μg. C3(H_2O), which has been shown to possess C3b-like functional properties (23), was obtained by repeated cycles of freezing (at −20°C) and thawing of the purified protein and was inactive in a C3 hemolytic assay.

Treatment of bacteria and quantitation of bound C3. Bacteria (0.5×10^8 to 1×10^8/ml) were incubated in 2.5 to 80% (vol/vol) NHS at 37°C for different times, as indicated, with periodic agitation. NHS was used without prior treatment or was made 5 mM in $MgCl_2$–3 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (Mg^{2+}EGTA) to limit complement activation to the AP. For controls, NHS was inactivated at 56°C for 30 min or treated with 5 mM EDTA to block both complement activation pathways. Treated bacteria were harvested by centrifugation at 13,000 × *g* for 10 min at 4°C, washed three times in phosphate-buffered saline (PBS), and suspended to a concentration of 4×10^7/ml, estimated spectrophotometrically. In some experiments, bacteria were incubated with purified C3 or C3(H_2O) to investigate *Listeria* intrinsic C3-cleaving activity.

For quantitating C3 binding, 0.1-ml aliquots of suspension containing about 4×10^6 treated bacteria were dispensed into wells of a 96-well vinyl microtiter plate and allowed to evaporate to dryness at 37°C overnight. The wells were blocked with 3% (wt/vol) bovine serum albumin in PBS for 1 h, and saturating levels (10 μg/ml) of a rat monoclonal antibody to C3d, clone 3, donated by P. Lachman (Medical Research Council Centre, Cambridge, United Kingdom) were applied to replicate bacterium-coated and control wells for 1 h. The wells were washed with PBS and incubated for 1 h with ^{125}I-labelled goat F(ab')$_2$ anti-rat immunoglobulin (Jackson Immuno Research Laboratory) (2×10^5 cpm per well; specific activity, 4×10^7 cpm/μg). After final washes, individual wells were cut out and counted for γ radioactivity. Background values for nonspecific binding were determined with untreated bacteria and subtracted.

Extraction and analysis of bound C3. Bacteria (10^8/ml) were incubated for 30 min at 37°C in 20% Mg^{2+}EGTA-chelated NHS supplemented with ^{125}I-labelled C3 (2% of endogenous C3). They were then washed three times with PBS containing 5 mM di-isopropylfluorophosphate (to minimize further proteolytic cleavage of C3). Washed bacterial pellets (5×10^5 cpm each) were resuspended in either 1 M hydroxylamine (pH 9.5) (to disrupt ester bonds), 100 mM dithiothreitol (DTT) (to disrupt disulfide bonds), 1% sodium dodecyl sulfate (SDS) (to remove noncovalently bound counts), or PBS (to assess spontaneous release, as described by Venkatesh et al. [27]) in a total volume of 0.5 ml and incubated for 30 min at 37°C. After centrifugation at 13,000 × *g* for 10 min, the supernatants were collected and the radioactivity was counted. The yield of total bound radioactivity extracted was calculated for each supernatant. Samples containing 10^4 cpm were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 7.5% acrylamide gel under reducing conditions, followed by autoradiography. The amount of C3b and iC3b was quantified by direct counting of the radioactivity on dried gels, by using a PhosphorImager (Molecular Dynamics). Controls for ^{125}I-C3 and ^{125}I-C3b were obtained by proteolysis of native C3 with 0.1% (wt/wt) trypsin for 5 min at 37°C.

C3 hemolytic activity. The residual hemolytic activity of purified C3 and of C3 in NHS after incubation with the various *Listeria* strains was measured by a one-step method using methylamine-treated plasma as a complement source and rabbit erythrocytes as indicator cells (18). Functional AP and classical pathway activity was determined in kinetic assays using rabbit erythrocytes and sensitized sheep erythrocytes, respectively (21).

The production of factor B fragments, Ba and Bb, upon incubation of strain EGD in NHS was analyzed by immunoelectrophoresis (12). Controls with NHS and Mg^{2+}EGTA NHS, but without bacteria, were performed in each test.

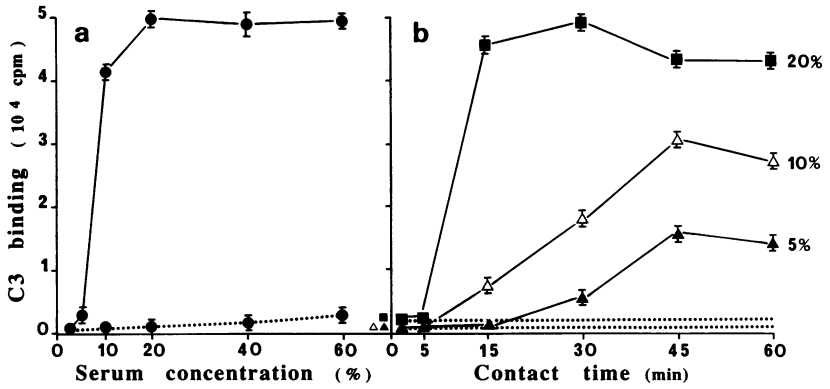

FIG. 1. Deposition of C3 onto strain EGD as a function of (a) the serum concentration used for a 30 min-incubation period and (b) the contact time of serum and bacteria. NHS was treated with EDTA (dotted line) or Mg^{2+}EGTA (continuous line) and adjusted to the indicated concentration before incubating with bacteria. Results are typical of three experiments.

RESULTS

Activation of the AP of human complement by *L. monocytogenes* EGD. The ability of *L. monocytogenes* EGD to produce AP activation during incubation with untreated nonimmune serum, NHS, was demonstrated by the selective reduction in AP functional activity, whereas classical pathway activity was virtually unaffected (not shown). AP activation by *L. monocytogenes* was confirmed by the capability of this organism to cause factor B cleavage in NHS (not shown).

The resulting C3 deposition on the bacterial surface was quantitated by a whole-cell radioimmunoassay with a rat monoclonal antibody to C3d. In preliminary experiments, the optimal amount of bacteria for this assay was determined by dispensing 0.1 ml of doubling dilutions from serum-treated strain EGD per well. At bacterial concentrations below 8×10^7/ml, the values of bound C3 varied in a dose-dependent manner and reached a plateau at higher concentrations, probably as a result of limitation of anti-C3 antibody binding due to steric hindrance. Therefore, a concentration of about 4×10^7 bacteria per ml was selected in subsequent experiments.

The influence of serum concentration on C3 binding, as well as its kinetics, is illustrated in Fig. 1. It is shown that C3 binding reached a plateau at concentrations up to 20% of NHS (Fig. 1a). At an NHS concentration of 20%, C3 binding was maximum in 15 min (Fig. 1b). Table 2 compares different reagents for the extent of C3 binding. C2-deficient serum and NHS, either untreated or chelated with Mg^{2+}EGTA (to prevent potential activation of the classical pathway by chelating Ca^{2+}), were similarly efficient with respect to C3 deposition. In contrast, methylamine-treated NHS supported background binding, comparable to that obtained with EDTA-chelated or heat-inactivated NHS. In the presence of methylamine-treated NHS, binding was totally restored upon addition of purified C3, demonstrating the requirement for an intact thioester in the C3 molecule for its binding to occur. Using purified C3 instead of serum led to a low level of binding, not different from that observed with its inactivated form, $C3(H_2O)$. Furthermore, C3 hemolytic activity was not altered upon incubation of bacteria with native C3, indicating the absence of intrinsic C3-cleaving protease. Taken together, these results indicate that C3 deposition onto the surface of *L. monocytogenes* results from AP activation, which does not lead to the bacterial lysis, as assessed by plate colony counting (not shown).

TABLE 2. Effects of different sources and treatments of serum and of purified C3 on deposition of C3 fragments on strain EGD

20% serum [treatment] and/or complement[a]	C3 binding[b]	
	cpm (mean ± SD)	% of NHS value
NHS [EDTA]	1,743 ± 260	4
NHS [none]	46,869 ± 980	100
NHS [Mg^{2+}EGTA]	44,397 ± 2,516	95
C2 deficient [Mg^{2+}EGTA]	40,573 ± 465	86
NHS [methylamine]	2,918 ± 630	6
NHS [methylamine] + C3	40,195 ± 2,112	86
C3	7,163 ± 450	15
$C3(H_2O)$	7,655 ± 350	16

[a] 30-min incubation. One hundred sixty-seven micrograms of complement per milliliter was used.
[b] In a representative experiment (n = 3).

C3 deposition and consumption by various strains of *L. monocytogenes* and related species. Various strains of *L. monocytogenes* and related species were compared for AP activation by assaying both C3 deposition at the membrane level and consumption of C3 hemolytic activity in the serum supernatant (Fig. 2). Except for the rough mutant (M8), the other strains tested bound similar amounts of C3 (mean ± standard deviation, [107.6 ± 10.5]% of EGD values) when incubated with 20% Mg^{2+}EGTA-chelated NHS. All strains, including those belonging to the pathogenic or nonpathogenic related *Listeria* species, led to similar C3 consumption that averaged (31.3 ± 3.9)% of total C3 activity. Mg^{2+}EGTA chelation of serum by itself (i.e., in the absence of bacteria) did not lead to C3 consumption.

To search for a component(s) on the bacterial surface involved in AP activation and/or resulting C3 deposition, the influence of various treatments of strain EGD (R1) in the above assays was studied. Heat killing and protease treatment of these bacteria did not alter C3 binding and consumption. In contrast, a 4-h culture with penicillin to produce cell wall-deficient bacteria reduced C3 binding by two-thirds while slightly lowering C3 consumption (Fig. 2).

Mode of binding and molecular form of bound C3. We then studied the release of deposited C3 on strain EGD pretreated with NHS (20%) added with low amounts of ^{125}I-C3. The percentages of deposited C3 released by treatments with

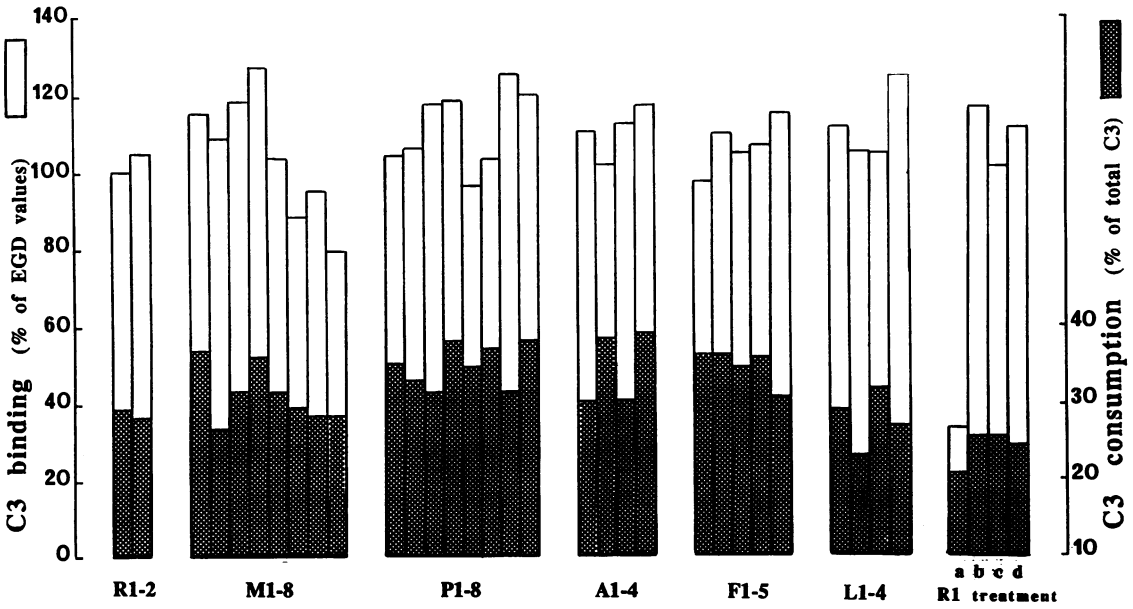

FIG. 2. Deposition and consumption of C3 during a 30 min-incubation of 20% Mg^{2+}EGTA-chelated NHS with the *Listeria* strains listed in Table 1: *L. monocytogenes* reference strains (R1 and -2), mutants (M1 to -8), and wild strains isolated from patients (P1 to -8), animals (A1 to -4), or food (F1 to -5); related species (L1 to -4); and *L. monocytogenes* EGD submitted to treatment with penicillin (a), pronase (b), or trypsin (c) or to heat killing (d). The standard deviation for 2 to 4 separate experiments did not exceed 5% for C3 consumption and 14% for C3 binding.

SDS (to release noncovalently bound molecules), DTT (to disrupt disulfide bonds), and PBS (to assess spontaneous release) were estimated to be 34.4, 13.5, and 8.5%, respectively. The total number of covalently bound C3 molecules (both labelled and unlabelled) retained on 20% NHS-treated *L. monocytogenes* after incubation with SDS was estimated to be about 1.3×10^5 per bacterium. To determine the type of linkage formed in the C3-acceptor molecule complex, we took advantage of the lability of the O-linked ester bond to hydroxylamine at an alkaline pH, whereas the amide bond is not susceptible to such an attack. An average of (52.4 ± 14.8)% (mean ± standard deviation) of the covalently bound radioactivity was released by hydroxylamine, with no significant difference between 27 strains of *L. monocytogenes* and the 4 strains belonging to the related species, indicating that opsonic deposition of C3 comprises both amide- and ester-linked species (data not shown).

The molecular forms of bound ^{125}I-C3 that could be released by the four different buffers (i.e., hydroxylamine, SDS, DTT, and PBS) were analyzed by SDS-PAGE and autoradiography. Results from a representative experiment are shown in Fig. 3. The material released from strain EGD by hydroxylamine comprised 54% C3b, 26% iC3b, and 20% C3d (Fig. 3, lane 2), and this pattern was not significantly modified as the time of contact between serum and bacteria increased from 15 to 90 min. Interestingly, the extent of conversion of bound C3b to iC3b plus C3d was reproducibly higher by one-third on the rough mutant compared with strain EGD. On L forms of EGD, the proportions of C3b, iC3b, and C3d were the same as those on the untreated strain, although in reduced amounts (data not shown). As expected, noncovalently bound C3 (released by SDS) possessed an intact α chain (Fig. 3, lane 1), whereas the small amounts of C3 released by DTT (putatively disulfide linked) exhibited a cleaved α′ chain (Fig. 3, lane 3). The release phenomenon occurring in PBS has been shown by Venkatesh et al. (27) to be exclusive for ester-linked complexes and to be abolished by SDS, explaining both the cleaved α′ chain in PBS-released C3 (Fig. 3, lane 4) and the exclusive presence of intact C3 in the SDS supernatant. The high-molecular-weight material in Fig. 3 (lanes 1 to 4) is likely to

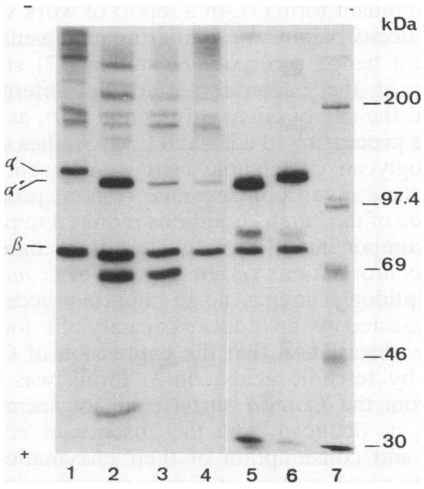

FIG. 3. Autoradiogram of an SDS-polyacrylamide gel (7.5% polyacrylamide) under reducing conditions, showing the molecular forms of C3 released from strain EGD treated for 30 min in 20% serum upon incubation with SDS (lane 1), hydroxylamine (lane 2), DTT (lane 3), or PBS (lane 4). Lanes 5 and 6, C3b and C3 controls; lane 7, molecular mass markers. The presence of the 105-kDa α′ chain indicates C3b, that of the 68-kDa chain indicates iC3b, and that of the 34-kDa chain indicates C3d. The 75-kDa band is the β chain common to both C3b and iC3b. The C-terminal piece of 37 kDa from the α′ chain of iC3b is not seen because it is poorly labelled by the iodination procedure.

reflect minor contamination of the tested supernatants by components of the bacterial surface complexed with C3 fragments.

DISCUSSION

Direct evidence is presented in this article that *L. monocytogenes* activates the AP of human complement, leading to binding to their membrane of C3b and its cleavage products, iC3b and C3d, through both ester and amide linkages. The same results were obtained with the four strains belonging to the related species *L. ivanovii*, *L. innocua*, *L. seeligeri*, and *L. welshimeri*. No difference could be detected according to the origin of *L. monocytogenes* strains, i.e., human, animal, or environment. With the exception of the rough mutant and L forms, the different strains of *L. monocytogenes* tested, including seven well-defined transposon-induced mutants, had similar behaviors regarding C3 binding to the bacterial surface and C3 consumption in the serum supernatant. Thus, the surface molecules binding C3b or regulating complement activation are not altered in the transposon mutants tested. This means that membrane-associated bacterial proteins such as ActA or internalin are not involved in these processes. This is not surprising, since ActA protein plays a role in the cellular cytoplasm to induce actin polymerization (19) and internalin is most suspected to directly interfere with unknown receptors on the surface of the cells, thus triggering internalization (10). In spite of the structural homology between internalin and the M protein of *Streptococcus pyogenes* (10), which inhibits C3 binding to the organism as a consequence of its affinity for factor H (7, 16), this membrane protein of *L. monocytogenes* clearly does not interfere with complement deposition.

Activation of the AP by *L. monocytogenes* is in agreement with reports on other gram-positive bacteria, and those quantitating the two forms of C3, C3b and iC3b, covalently bound to the bacterial surface have generally shown that C3b is the predominant form (7). In a report of work with strains of *Streptococcus pneumoniae* differing in capsular polysaccharides and hence serotype, Hostetter (17) stressed the importance of the bacterial surface in determining the amount and the site of covalently bound C3b, as well as its degradative processing to iC3b and C3d. Studies on whether the peptidoglycan or teichoic acid is responsible for AP activation have given controversial results, probably as a consequence of the harsh conditions required to purify these cell wall components that may alter their native linking molecular conformations (7). In the case of *L. monocytogenes*, the peptidoglycan may be an important acceptor structure, as suggested by the reduced capacity of L forms to bind C3 and the observation that the expression of O antigens, supported by teichoic acids, on L forms was unaltered. Proteins from the *Listeria* surface do not seem to play a major role, as deduced from the absence of effect on C3 deposition and consumption of their enzymatic stripping, although the rough mutant, which is strongly altered in the protein structure of the cell wall (14), proved to be less effective than strain EGD in activating the AP with higher susceptibility of deposited C3b to proteolytic processing. The lack of correlation between C3 deposition and consumption, obvious with L forms, had already been noted by Blaser et al. (4) with *Campylobacter fetus*. Also in keeping with previous reports concerning gram-positive bacteria is the resistance of *L. monocytogenes* to complement-mediated lysis, occurring probably because the thick cell wall prevents access of C5-C9 to the inner membrane (7). The killing of *L. monocytogenes* by fresh rat serum has been shown by Davies et al. (8) to involve the release of a β-lysin by platelets during blood clotting.

Our results extend previous observations regarding the opsonization of *L. monocytogenes* by serum (2, 5, 13, 20, 24, 26). Several studies have suggested that heat-labile opsonic factors from serum, presumably complement, may participate with macrophages in the clearing of *L. monocytogenes* from infected foci (9, 13, 29). In the present study, the capability of this pathogen to activate solely the AP was examined in the setting of undetectable specific antibodies. This does not preclude a role for the classical pathway of complement activation in *Listeria*-immune individuals. The relative contributions of antibodies and complement to *L. monocytogenes* opsonization are unclear. For example, Peterson et al. (24) and MacGowan et al. (20) have shown that immunoglobulin G in NHS was the major requirement for opsonization of *L. monocytogenes* and that heat inactivation of serum did not alter its opsonic activity. In contrast to their results, Bortolussi et al. (5) found that, when NHS is used at a concentration as low as 3%, opsonization of *L. monocytogenes* requires both immunoglobulin M and complement, primarily an intact classical pathway. Finally, using mouse serum and homologous peritoneal macrophages, Drevets and Campbell (9) found that the majority of phagocytosis of opsonized *L. monocytogenes* was abrogated by heat inactivation of the serum or preincubation with anti-CR3 antibody. The disparity between the above results may be explained by variability in the experimental conditions, especially serum concentration and animal origin, immune status of the serum donor, and type of phagocytic cells used.

Further investigations are needed to delineate the functional significance of our observations. In particular, insight will be gained into the relative contributions of complement opsonization and the bacterial surface protein internalin to *L. monocytogenes* entry into mononuclear phagocytes, as well as the putative influence of covalently associated C3 fragments on the intracellular fate and processing of bacterial antigens.

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