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Complement Activation by Polysaccharide of Lipopolysaccharide: An Important Virulence Determinant of Salmonellae

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Salmonellae with differences only in the O-antigenic polysaccharide of their lipopolysaccharide were previously shown to differentially activate complement via the alternative pathway, causing them to be ingested at different rates by the mouse macrophage-like cell line J774. We now show that this mechanism could explain the different virulence of these strains in vivo. Mouse peritoneal macrophages (thioglycolate induced) ingest these salmonellae at rates that are inversely proportional to the known virulence of the organisms and virtually identical to the rates observed with J774. As with J774, complement is required for this differential uptake, since serum was required and heating (56°C for 30 min) or zymosan treatment of the serum destroyed activity. The known receptor for nonreducing terminal mannose-, fucose-, *N*-acetylglucosamine, and glucose-containing glycoproteins did not participate, since uptake was not inhibited by high concentrations of mannan. When clearance of bacteria from the bloodstream of mice was measured, the least virulent organism was cleared very much faster than the most virulent organism, in confirmation of earlier data. When complement in the mice was destroyed by pretreatment with cobra venom factor, the clearance of the least virulent strain was greatly reduced, whereas the very slow clearance of the most virulent strain was unaffected. These data strongly support the hypothesis that when bacteria have polysaccharide in lipopolysaccharide that activates complement efficiently, the bacteria will be phagocytosed, whereas if the polysaccharide activates complement poorly, the bacteria escape ingestion and may cause disease.

Salmonella strains differing only in the carbohydrate side-chain of their lipopolysaccharide (LPS) differ in their virulence for mice (25, 51, 52, 54). These strains were constructed by transduction and recombination of *Salmonella typhimurium* with genes of the *rfb* gene cluster coding for antigenic side chains in LPS (Table 1). We found that the rate of phagocytosis of such strains by the mouse macrophage-like cell line J774 is inversely proportional to virulence (22) and results from differential activation of complement by these bacteria via the alternative pathway (21a).

It is therefore tempting to speculate that the differential virulence of these strains in vivo results from the differential uptake of the strains by macrophages via their C3b receptor. However, the previous results were insufficient to prove this hypothesis.

First, the mouse macrophage-like cell line used differs from macrophages derived directly from mice; for instance, it has been found (45) to lack the receptor for exposed nonreducing terminal mannose, fucose, *N*-acetylglucosamine, or glucose which is present in mouse-derived macrophages (46). Since mannose, *N*-acetylglucosamine, and glucose occur in the antigenic polysaccharides of these strains (Table 1), one might postulate that in vivo uptake proceeds via this receptor, rather than the C3b receptor. Cell line J774 might also differ from mouse-derived macrophages in other important, but as yet undiscovered, ways.

Second, even if mouse peritoneal macrophages ingest via the same mechanism as does the cell line J774, this mechanism does not necessarily operate in infected mice. Although it is known that when these strains are injected

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into mice their clearance is inversely proportional to their virulence (48). suggesting that phagocytosis in vivo, as in vitro, depends on polysaccharide of LPS, there might be mechanisms of clearance other than complement-dependent phagocytosis. Therefore, it was necessary to determine whether clearance was dependent on complement.

In the present work we show that thioglycolate-elicited macrophages take up these *Salmonella* strains at rates virtually identical to those observed with J774, and that the uptake is dependent on complement, rather than the above-mentioned carbohydrate receptor. Furthermore, when mice are depleted of complement by treatment with cobra venom factor, the rapid clearance of the least virulent strain is slowed, whereas the very slow clearance of the most virulent strain is unaffected, indicating that the complement-catalyzed phagocytosis first described in vitro is an important host defense mechanism in vivo.

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MATERIALS AND METHODS

Supplies and reagents. Cell culture reagents were as follows: fetal bovine serum was from Reheis Chemical Co. (Kanakee, Ill.); α-Eagle minimum essential medium and L-glutamine (200 mM) were from M-A Bioproducts (Walkersville, Md.); trypan blue (0.4%), penicillin (10^4 U/ml), and streptomycin (10^4 μg/ml) were from GIBCO Laboratories (Grand Island, N.Y.); cobra venom factor (CVF) from *Naja haje* was from Cordis Laboratory (Miami, Fla.); and zymosan was from Schwarz-Mann, Inc. (Orangeburg, N.Y.). Zymosan-treated serum was prepared by incubating serum with activated zymosan (20 mg/ml) by the method of Pillemer et al. (32). *Vibrio cholerae* neuraminidase was from Calbiochem (La Jolla, Calif.), and the nonspecific esterase stain was from Technicon Instrument Co. (Tarrytown, N.Y.). Mice were from the National Institutes of Health small animal section. The mouse macrophage-like cell line J774 (36, 37) was originally provided by Peter Ralph (Sloan-Kettering Institute for Cancer Research, Rye, N.Y.).

Preparation of macrophages. Peritoneal macrophages were harvested and maintained in culture by a modification of the method of Cohn and Benson (7). Briefly, mice were injected intraperitoneally with 1 ml of Brewer thioglycollate medium (England Laboratory, Beltsville, Md.); macrophages were harvested by peritoneal lavage 4 days later, washed with medium, applied to a 13 mm diameter glass cover slips, and incubated in complete medium. The cover slips were washed 2 h later to remove nonadherent cells, incubated for 16 h, washed again, and used at once. The viability of adherent cells was >95%, as measured with trypan blue. Adherent cells were demonstrated to be 85% macrophages by nonspecific esterase-lipase stain (47) and by their ability to ingest latex beads.

The J774 cell line was maintained and plated on cover slips exactly as described previously (32).

Preparation of bacteria. The *Salmonellae typhimurium* transductants and recombinants used in these studies have previously been described (5, 11, 48) (Table 1), and their conditions of growth, labeling by growth for several generations in [^{14}C]glucose, and fixation with glutaraldehyde were as previously described (22).

Phagocytosis in vitro. Labeled bacteria were added in medium to macrophages on coverslips, and the uptake of bacteria with time was measured as previously described (22). As demonstrated previously by fluorescent microscopy (22), the bacteria taken up are in the macrophages; virtually no uptake or adhesion occurs at 4°C (binding via the complement receptor does not occur at 4°C). The uptake reported herein equals the radioactivity at 37°C minus the radioactivity at 4°C (see Fig. 1 of reference 22).

Phagocytosis in vivo (clearance test). The clearance test (21) was performed by injecting 0.2 ml (about 10^8 bacteria) of an exponentially growing broth culture

TABLE 1. Derivatives of *S. typhimurium* and their 50% lethal doses after intraperitoneal injection into mice

LPS side chain structure determined by *rfb* genes originally from:	LPS side chain antigenic specificity	Structure of LPS side chains	Strain	50% Lethal dose
S. typhimurium	O-4,12	Abe | (Man-Rha-Gal)n[a]	SH5771[b]	2×10^4
S. enteritidis	O-9,12	Tyv | (Man-Rha-Gal)n[a]	SH4340[c]	5×10^4
S. montevideo	O-6,7	Glu / \ (Man-Man-Man-Man-GlcNac)n[d]	SH5770[b]	1×10^6

[a] From reference 29.
[b] From reference 52. These two strains are paired recombinants.
[c] From reference 51. This strain is a transductant of an originally O-4,12 strain.
[d] From reference 11.

TABLE 2. Uptake by thioglycollate-elicited macrophages and cell line J774

LPS side chain	Rate of uptake (bacteria per macrophage per h)[a]		
	J774	C3H/HeN	C57BL/6
O-6,7	17.1	14.5	16.5
O-9,12	7.7	5.1	5.8
O-4,12	2.9	3.1	1.7

[a] Conditions were as for Figure 1. Incubation was for 3 h.

into the central tail vein of heparinized mice (outbred approximately 8-week-old). Blood samples (0.05 ml) were taken after 0.5, 1, 2, 3, and 5 min from the retroorbital venous plexus and mixed with 5 ml of NaCl (0.9%). Blood samples were plated immediately in suitable dilutions for enumeration of viable bacteria. The clearance rates of the bacteria were calculated from these data by linear regression analysis. Three mice were used for each data point. The significance of the differences of the slopes was tested with the Student *t*-test.

CVF treatment. Lyophilized CVF was reconstituted in cold distilled water and diluted in phosphate-buffered saline. Animals were given 5 U of CVF in a volume of 200 μl intravenously at 0 and 48 h. They were used at time 72 h for the clearance test and the assay of hemolytic complement.

Complement microassay. Serum was obtained from the mice from the retroorbital venous plexus. The blood of five separate mice was pooled, allowed to clot in an ice-water bath for 60 min, and centrifuged at 1,000 × *g* for 10 min at 4°C. The serum was kept at −20°C until use on the following day.

A microassay for the mouse alternative pathway hemolytic activity was used (20). In brief, rabbit erythrocytes [5 × 10^8 erythrocytes per ml in magnesium ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid buffer] were incubated for 2 h with neuraminidase at 0.8 U/ml, washed, and labeled with $Na^{51}CrO_4$. The hemolytic assays were performed in 1.5-ml microcentrifuge (Eppendorf) tubes containing 50 μl of chromium-labeled, neuraminidase-treated rabbit erythrocytes and 200 μl of magnesium ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid buffer or various dilutions of mouse serum in the buffer. Chromium release into the supernatant was measured after 1 h of incubation at 37°C. Control tubes contained 200 μl of water (100% lysis) or buffer (spontaneous chromium release).

RESULTS

Phagocytosis of bacteria by mouse peritoneal macrophages. The rate of uptake by thioglycollate-elicited mouse peritoneal macrophages of bacteria bearing O-4,12, O-9,12, and O-6,7 polysaccharide on LPS is shown in Fig. 1A. The rates were almost identical to those previously demonstrated with the mouse macrophage-like cell line J774 (Table 2) and did not differ when two strains of mice, one very sensitive and one much less sensitive to *S. typhimurium* infection, were compared (34). As with the cell line (32), the most virulent strain of *S. typhimurium* (O-4,12) was taken up slowest, the one of intermediate virulence (O-9,12) was taken up at an intermediate rate, and the least virulent (O-6,7) was taken up fastest.

When heated serum (56°C for 30 min) or zymosan-treated serum was substituted for normal serum (Fig. 1B and C), the uptake of O-6,7 and O-9,12 was greatly reduced. The uptake of O-4,12, which was already low, was not perceptibly reduced. These results suggest that complement is required for the observed differences in uptake, and that in its absence all three strains are taken up at the same, low rate. When serum was omitted, the results were identical to those observed with heated or zymosan-inactivated serum (data not shown). The same results were obtained previously with the cell line J774 (22).

Since the LPS produced by the least virulent strain contains nonreducing terminal mannose and glucose (Table 1), which might be a substrate for the macrophage carbohydrate receptor (46), uptake was examined also in the presence of yeast mannan, an inhibitor of the carbohy-

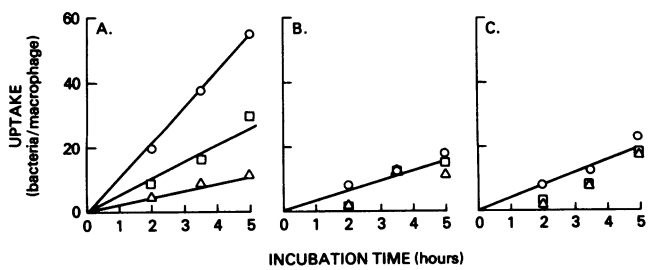

FIG. 1. Uptake of salmonellae differing in polysaccharide of LPS by thioglycollate-elicited macrophages from C3H/HeN mice. Macrophages (3 × 10^5 per cover slip) and bacteria (2 × 10^8/ml) were incubated at 37°C in medium containing (A) 10% fetal bovine serum, (B) 10% heated fetal bovine serum (56°C, 30 min), or (C) 10% zymosan-treated fetal bovine serum. Symbols: salmonellae with O-6,7 polysaccharide (○); O-9,12 polysaccharide (□); O-4,12 polysaccharide (△).

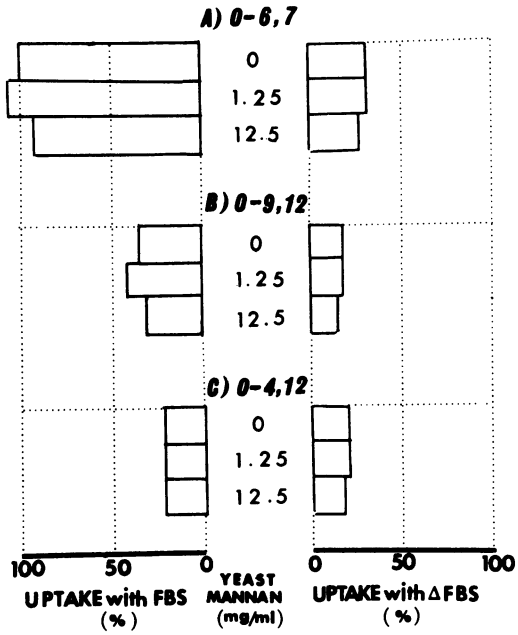

FIG. 2. Effect of mannan on uptake of bacteria by thioglycollate-elicited macrophages from C57BL/6 mice. Conditions were as for Table 2. Mannan was added at the indicated concentrations. The uptake of O-6,7 bacteria without mannan was set as 100%. (The numerical values for rates without mannan are given in Table 2.)

drate receptor. Yeast mannan at concentrations of up to 12.5 mg/ml did not inhibit the uptake of all three bacteria (Fig. 2); this is 10 times the concentration of mannan that fully inhibits binding of mannose-containing proteins by the carbohydrate receptor of mouse macrophages (46). To determine whether the residual uptake which remained in the absence of serum or the presence of heated serum might proceed via this receptor, mannan was also added to cells incubated with bacteria in heated serum (Fig. 2). The results show that there is no inhibition of the residual uptake by mannan. Thus, the carbohydrate receptor is unlikely to participate in uptake of these bacteria.

Clearance of bacteria in vivo and its dependence on complement. Salmonellae containing O-6,7 antigen were removed from the blood of mice more rapidly (slope of the line representing clearance rate, −0.267) than were the more virulent O-4,12 bacteria (slope, −0.001) (Fig. 3), in accordance with previous results (48). To determine whether this clearance required complement, mice were treated with CVF, which causes depletion of alternative complement pathway components including C3 (6, 19, 24, 31). After such treatment, their ability to clear the O-6,7 bacteria was significantly reduced (slope, −0.05) (Fig. 3). These results show that the rapid clearance of the O-6,7 bacteria is dependent on an intact alternative pathway.

To determine the degree of reduction of complement levels by this CVF treatment, the activity of the alternative pathway in treated and untreated mice was measured by an assay based on the ability of neuraminidase-treated rabbit erythrocytes to be lysed by complement in the absence of antibody (19, 20, 35). Half-maximal lysis was achieved by normal mouse serum at a dilution of 1:32, under conditions in which undiluted serum from the mice treated with CVF caused no lysis. Thus, CVF treatment had reduced the alternative pathway activity of complement to less than 3% of normal levels.

DISCUSSION

The rate of uptake of salmonellae strains by the mouse macrophage-like cell line J774 depends on the polysaccharide of their LPS (22) and reflects differential activation of complement via the alternative pathway (21a). It was attractive to speculate that this mechanism oper-

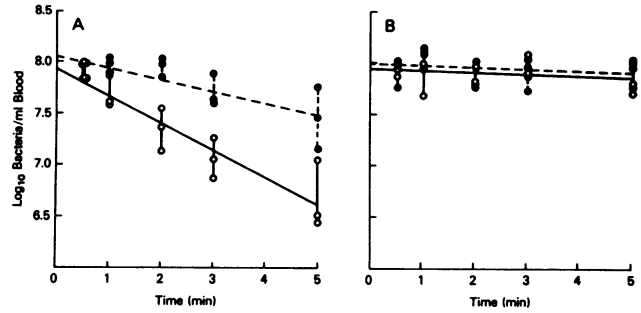

FIG. 3. Clearance of salmonellae with O-6,7 (A) or O-4,12 (B) polysaccharide from the blood of untreated (○) and CVF-treated (●) mice. The vertical bar between the circles represents the scatter of data from three mice. The slopes were calculated by linear regression analysis.

ates in vivo since the differences in phagocytosis are in the right direction to explain the differences in virulences among these strains (25, 51, 52, 54) and also parallel previously observed differences in the ability of the mice to remove the bacteria from their bloodstream. Thus, the O-4,12 bacteria were cleared much more slowly than were the O-6,7 strains (48), by a mechanism believed to involve resident macrophages in the liver and, to a lesser extent, in the spleen and lungs (3). The removal of the O-6,7 bacteria did not require antibody, since no anti O-6,7 antibodies could be detected and the differential virulence was manifested even in animals unable to make antibody due to X-irradiation and thymectomy or cyclophosphamide treatment (49, 53).

There is a complex interplay between many bacterial and host factors responsible for production of disease (23, 30). Bacterial virulence can be reduced not only by changes in LPS, but also by mutations to auxotrophy for nutrients not readily available in the host (1, 2, 23, 44), mutational loss of enterochelin (55), loss of fimbriae (8), and loss of enterobacterial common antigen (50). Host virulence factors include those specified by the *Ity* (33, 34), the *xid* and the *lps* loci (see references 30 and 42 for reviews). Recent evidence indicates that the *Ity* locus controls the rate of multiplication of bacteria after ingestion by phagocytic cells, such that Ity^r mice prevent multiplication of ingested bacteria better than do Ity^s mice (14). The present studies confirm that the *Ity* locus may work at a stage of infection other than ingestion, since rates of uptake of these bacteria by macrophages from C57B1/6 (Ity^s) and C3H/HeN (Ity^r) mice were comparable (Table 2), although these mice differ by several orders of magnitude in their sensitivity (by 50% lethal dose) to a given salmonella (34). We may postulate that the structure of bacterial LPS can determine complement activation and therefore ingestion, but the fate of the internalized bacteria would depend on the host *Ity* locus.

The current results indicate that ability of these bacterial strains to trigger complement-mediated phagocytosis affects their relative virulence. First, the differential uptake first observed in the macrophage-like cell J774 is also shown by peritoneal macrophages and involves the complement receptor rather than the receptor for reducing terminal mannose, glucose, and glucosamine (46). Second, destroying complement in vivo with CVF lowers the rate of clearance of the O-6,7 bacteria, whereas the low rate of clearance of the O-4,12 bacteria is unaffected. Thus, the rapid clearance of the less virulent bacteria requires complement both in vivo and in vitro. Parenthetically, the O-4,12 bacteria can be cleared if opsonized by another method: if coated with anti-LPS antibodies they are cleared rapidly in vivo, and their ability to cause infection is reduced (4, 48). Furthermore, this antibody-enhanced phagocytosis appears to require complement since it is slowed in C3-depleted mice (manuscript in preparation).

Previous data suggested a role for complement in the clearance of bacteria independent of complement lysis. Thus, serum-resistant organisms are efficiently cleared, indicating that factors other than bactericidal activity are important in such clearance (41), and *S. typhi* could be cleared at a normal rate in C6-deficient rabbits (43). Nevertheless, the role of complement in clearance in nonimmune animals was questioned (27), partially because, although C3b receptors are reported to mediate phagocytosis by polymorphonuclear leukocytes (12), monocytes (16), and activated macrophages, resident nonactivated peritoneal macrophages do not ingest, although they do bind, via this receptor (2, 9, 13, 26). Despite these in vitro data, recent work of Frank and collaborators (5, 15) has clearly shown that complement activation by pneumococci results in their clearance in nonimmune animals. We now show that such nonimmune animals can also clear salmonellae that activate complement and further that this clearance is important in host defense.

The presence of O-side chains in LPS is known to be important in preventing complement killing. Rough strains lacking such side chains are killed by complement, but smooth strains possessing them are not (10); instead they are killed only when the LPS layer is disturbed, for instance by EDTA treatment (38–40). Smooth strains were recently found to be serum resistant because they bind the C5b-9 complex poorly, in contrast to effective tight binding by serum-sensitive strains (17, 18). Thus, prior data showed that the presence of O-side chains is important, but the importance of side chain saccharide structure was not fully appreciated. The evolution of the unusual sugars often found in such side chains was hypothesized to reflect selective pressure for antigenic diversity, to preserve the invading bacterium from antibodies synthesized in response to prior infection (28). We may now add another function of such diversity: these unusual sugars may mask determinants that otherwise would activate complement, triggering phagocytosis and possibly death of the organism. It is striking that the O-4,12 and O-9,12 saccharide structure differ only in the configuration of two carbon atoms on an unusual dideoxyhexose. Apparently this small difference engenders a large difference in complement activation, by mechanisms as yet unknown.

Studies both on the molecular mechanism of alternative pathway activation and on the phagocytic cells may provide further information on this mechanism of virulence and defense.

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