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Protective Role of Smooth Lipopolysaccharide in the Serum Bactericidal Reaction

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The mechanism by which smooth lipopolysaccharide (LPS) protects *Salmonella typhimurium* C5 from the lethal action of specific antibody and complement has been investigated. Tris(hydroxymethyl)aminomethane (Tris) and ethylenediaminetetraacetate (EDTA), which sensitize *S. typhimurium* C5 to the bactericidal action of immune serum, were shown to cause appreciable structural modification of the smooth LPS associated with these cells. Mg^{2+} was shown to reverse the sensitization of C5 by Tris, but this divalent cation was unable to reverse the sensitizing effect of Tris plus EDTA, which, unlike Tris, caused relatively large amounts of O-antigenic components to be released from the C5 cell wall. The possibility that smooth LPS blocks the interaction of activated complement components with receptors on the cell wall is discussed.

It has been demonstrated (15, 16) that smooth gram-negative organisms, which were completely insensitive to the bactericidal action of specific antibody and complement when tested in various salt solutions containing Mg^{2+} or Ca^{2+} (nonsensitizing media), became susceptible when the bactericidal assay was carried out in solutions of tris(hydroxymethyl)aminomethane (Tris) or Tris plus ethylenediaminetetraacetic acid (EDTA), both being sensitizing media. It was suggested (15) that the sensitizing systems may modify the structure and hence some protective function of the smooth lipopolysaccharide (LPS) layer of the outer cell wall.

The thick LPS layer of smooth gram-negative cells may give protection in any of the following ways: (i) antibody may be fixed so far from the bacterial cell wall proper that the activated components of complement are unable to interact with the cell in sufficient concentration to cause lethal damage (1, 5, 10, 16); (ii) complement-mediated damage occurs but is not lethal due to the supportive function of the thick LPS coat (5); (iii) the substantial polysaccharide outer layer inhibits the action of complement by blocking cell receptors to which activated components must bind in the reaction sequence which results in cell death.

In this paper we describe experiments designed to investigate these possibilities.

MATERIALS AND METHODS

Strains, antiserum, and bactericidal assays. A previous report describes strains, antiserum, and bactericidal assays used (15).

Preparation of LPS. LPS from *S. typhimurium* C5 was prepared by the phenol-water method of Westphal et al. (20) and purified by alcohol precipitation, enzymatic treatments to remove nucleic acids and protein, and finally by ultracentrifugation.

Sensitization of sheep red blood cells. To a 4% (v/v) suspension of washed sheep red blood cells in physiological saline, an equal volume of lipopolysaccharide was added at a concentration of 100 μg/ml. After 2 hours of gentle mixing at 37 C, the cells were washed three times in saline and resuspended in saline to a concentration of 0.5%.

Hemagglutination assays and inhibition studies. Serial 0.2-ml twofold dilutions of antiserum were prepared in Perspex trays. To each well, 0.2 ml of a 0.5% suspension of sensitized red cells was added, and the plates were incubated for 1 hr at 37 C. Results were read after standing overnight at 4 C. The end point was read as the highest dilution giving complete agglutination, and this dilution of antiserum was taken as equivalent to 1 hemagglutinating unit. Inhibition studies were carried out with 0.2-ml twofold dilutions of pure LPS and 0.1-ml amounts of antiserum containing the equivalent of 4 hemagglutinating units. After incubating these systems at 37 C for 30 min, 0.2 ml of a 0.5% suspension of red cells was added and the plates were incubated for 1 hr at 37 C. Hemagglutination was read after standing overnight at 4 C. Unknown amounts of hemagglutination-inhibiting material from C5 cells were determined by comparison with the inhibitory effect of pure C5 LPS in the same system. This method enabled us to detect as little as 20 ng of pure LPS.

Radioactive labeling of glycopeptide. A 10-ml amount of an 18-hr-old broth culture of bacteria was diluted into 50 ml of nutrient broth at 37 C containing 50 μg of ^{3}H-diaminopimelic acid (DAP;

specific activity, 0.76 mCi/mg) and grown with mild aeration to about 5×10^8 cells per ml. These cells were washed three times in physiological saline and made up to 30 ml prior to experimental use. A 0.1-ml portion of the washed cell suspension gave about 20,000 counts/min by liquid scintillation.

Glycopeptide degradation. To measure acid-insoluble radioactivity, 0.1-ml samples from experimental tubes were transferred to 2-cm discs of glass-fiber paper (Whatman GF3), which were immediately dropped into cold 5% trichloroacetic acid. The discs were washed with cold 5% trichloroacetic acid, washed again with ethanol, and dried in a vacuum oven. The samples were counted in a Packard liquid scintillation counter with 5 ml of scintillant [toluene, 1 liter; 2,5-diphenyloxazole, 4 g; 1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene, 0.5 g] with an efficiency for ^3H in DAP of 40%.

Intrinsic viscosity [η]. The viscosity of solutions of LPS was determined at 37 C in a zero-shear capillary viscometer, with a flow time for water of about 600 seconds. The viscosity (η) at five different concentrations of LPS, at 2 mg per ml and below, was determined relative to the viscosity of appropriate solvent (η_0). The relative viscosity ($\eta_r = \eta/\eta_0$) and hence the specific viscosity ($\eta_{sp} = \eta_r - 1$) was determined for each concentration of LPS (C) in a particular solvent. The intrinsic viscosity ($[\eta] = \lim_{C \to 0} \eta sp/c$) was determined by plotting in ($\eta sp/c$) against C and extrapolating to zero concentration.

Electron microscopy. LPS samples were placed on carbon-coated grids and negatively stained with 2% uranyl acetate.

RESULTS

LPS release from S. typhimurium C5. Tris plus EDTA has been shown to cause the rapid release of relatively large amounts of LPS from several gram-negative bacteria (8). We thought, therefore, that the imposed sensitivity to serum when C5 was suspended in Tris plus EDTA or in Tris alone might result from the stripping of smooth LPS from the outer surface of these bacterial cells. We therefore investigated the release of O-antigenic material (LPS) from C5 cells when incubated in solutions of Tris plus EDTA, in Tris alone, in physiological saline which has been shown to induce slight serum sensitivity in C5 (15), and in physiological saline plus Mg^{2+} (0.002 M) in which the cells were completely serum-resistant. The results of these experiments (Table 1) showed that, although relatively large amounts of LPS were released in Tris plus EDTA, very little was released in Tris alone, little more in fact than was released in physiological saline or in the completely nonsensitizing medium of physiological saline plus Mg^{2+}.

Reversibility of Tris sensitization. We had previously demonstrated that Mg^{2+} inhibited the sensitization of C5 by Tris (15). The data presented in Table 2 showed that bacterium/antibody complexes (BA) formed by adsorbing specific antibody to C5 cells in Tris were insensitive to complement when washed and resuspended in saline plus Mg^{2+}, although the same BA were very sensitive to complement when resuspended in Tris. Thus Tris sensitization could be reversed by Mg^{2+}. Also, BA formed in saline plus Mg^{2+}, although resistant to complement action when

TABLE 1. *Release of lipopolysaccharide (LPS) from Salmonella typhimurium C5 incubated in various salt solutions*

Salt solution[a]	Amt (μg) of LPS released per mg (dry weight) of bacteria
Tris (0.1 M; *p*H 7.4) + EDTA (200 μg/ml)	38
Tris (0.1 M; *p*H 7.4)	1.6
Physiological saline	1.2
Physiological saline + Mg^{2+} (0.002 M)	0.8

[a] Equal numbers of bacteria (10^{10} cells per ml) were suspended in each of the solutions specified and incubated at 37 C for 1 hr. The hemagglutination-inhibiting ability of each supernatant fluid was determined and compared with that obtained with a pure sample of C5 LPS. Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

TABLE 2. *Effect of ionic environment on the adsorption of antibody and the bactericidal action of complement against Salmonella typhimurium* C5[a]

Antibody adsorbed in	Inoculum surviving (%) 60 min of incubation with 1:10 guinea pig serum in	
	Tris	Saline + Mg^{2+}
Tris	<1	188
Saline + Mg^{2+}	<1	196
Controls		
Tris[b]	168	200
Physiological saline + Mg^{2+}[b]	150	232
No antibody treatment[c]	162	187

[a] Bacteria (about 10^8 cells per ml) were suspended in tris(hydroxymethyl)aminomethane (Tris; 0.1 M; *p*H 7.4) or in physiological saline plus Mg^{2+} (0.002 M) and incubated with rabbit antiserum (1:500) for 20 min at 37 C. The cells were then washed in physiological saline and resuspended at about 5×10^4 cells per ml in the diluent at 37 C in which the assay was to be performed.

[b] Assay with heated guinea pig serum.

[c] Assay with normal guinea pig serum.

washed and resuspended in this medium, were very sensitive when resuspended in Tris. We presume therefore that the site at which antibody is fixed to the cell is not an important factor in determining C5 sensitivity.

Irreversible sensitization of C5 by Tris + EDTA. BA were prepared in either Tris or in saline plus Mg^{2+}, subsequently incubated with Tris plus EDTA, washed, and then resuspended in either Tris or saline plus Mg^{2+}. These cells proved very sensitive to complement in saline plus Mg^{2+} as well as in Tris (Table 3). Thus the complement sensitization induced by Tris plus EDTA was not reversible by Mg^{2+}, presumably because it involved the release of significant amounts of LPS.

TABLE 3. *Effect of Tris + EDTA treatment on the sensitivity of Salmonella typhimurium C5 to the bactericidal action of complement*[a]

Antibody adsorbed in	Inoculum surviving (%) 60 min of incubation with 1:10 guinea pig serum in	
	Tris	Saline + Mg^{2+}
Tris[b]	<1	150
Saline + Mg^{2+}[b]	4	165
Control[c]	146	
Tris[d]	<1	2
Saline + Mg^{2+}[d]	2	7
Control[e]	158	

[a] Bacteria (about 10^8 cells per ml) were suspended in tris(hydroxymethyl)aminomethane (Tris;0.1 M; *p*H 7.4) or in physiological saline plus Mg^{2+} (0.002 M) and incubated with rabbit antiserum (1:500) for 20 min at 37 C.

[b] Portions of cells washed in physiological saline and resuspended at about 5×10^4 cells per ml in diluent in which assay was to be performed.

[c] Bacteria incubated in Tris for 20 min at 37 C.

[d] As above, but portions of washed cells were incubated with Tris + ethylenediaminetetraacetic acid (EDTA; 200 μg/ml) for 10 min prior to final wash and resuspension at about 5×10^4 cells per ml in appropriate diluent.

[e] Bacteria incubated in Tris for 20 min at 37 C, followed by 10 min in Tris + EDTA (200 μg/ml).

TABLE 5. *Effect of ionic environment on the intrinsic viscosity of lipopolysaccharide from Salmonella typhimurium C5*

Solvent	Intrinsic viscosity [η]
0.005 M Mg^{2+}	10 (15.0–17.5 nm)[a]
Deionized water	100
EDTA (400 μg/ml)[b]	140
Tris (0.1 M)[b]	145 (7.5–12.0 nm)[a]
Tris (0.1 M) + EDTA (400 μg/ml)	400 (7.5–12.0 nm)[a]

[a] Diameter of lipopolysaccharide strands from electron micrographs (Fig. 1–3).

[b] EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

Correlation of glycopeptide degradation with cell death. The data presented so far are compatible with the suggestion of Feingold et al. (5) that smooth gram-negative cells may suffer damage by complement but recover due to the supportive nature of their thick LPS coat. However, the results presented in Table 4 are against this view. We examined the effect of guinea pig serum complement and additional lysozyme on the glycopeptide layer of C5 cells in Tris, which produced cell death, and in saline plus Mg^{2+}, which did not. The release of acid-soluble radioactive materia from the specifically labeled glycopeptide oc

TABLE 4. *Effect of serum on the degradation of glycopeptide, cell lysis, and cell survival with two strains of Salmonella typhimurium suspended in Tris or in saline + Mg^{2+}*[a]

Test organism	Per cent radioactivity released		Per cent fall in OD_{650}		Per cent killing	
	Tris	Saline + Mg^{2+}	Tris	Saline + Mg^{2+}	Tris	Saline + Mg^{2+}
S. typhimurium C5[a]	52	5	62	6	94	0
S. typhimurium M206[a]	72	53	69	10	99	97
S. typhimurium C5[b]	7	2	0	0	0	0
S. typhimurium M206[b]	17	15	2	3	0	0

[a] Bacteria (about 10^9 cells per ml) growth labeled with ^{3}H-DAP (diaminopimelic acid) in broth were washed in physiological saline at 37 C and resuspended in physiological saline at 37 C containing rabbit antiserum (1:500). After 20 min of incubation at 37 C, the cells were washed with physiological saline and resuspended in either tris(hydroxymethyl)aminomethane (Tris; 0.1 M; *p*H 7.4) or in physiological saline plus Mg^{2+} (0.002 M) at 37 C; guinea pig serum (1:10) and lysozyme (2.5 μg/ml) were then added to each system. The values tabulated were reached after 60 min of incubation.

[b] As above, except that guinea pig serum was heat-inactivated.

FIG. 1. *LPS in 0.005 M Mg^{2+}; negatively stained with 2% uranyl acetate.*

curred only in those cell populations which sustained lethal complement damage. Very little release of label was observed from cells which survived; these presumably escape significant complement damage to their outer lipid membrane.

The partly rough *S. typhimurium* M206 strain, which has been shown to be sensitive to complement in both Tris and in saline plus Mg^{2+} (15), lost large amounts of label when treated with complement plus lysozyme in each of these media, although the optical density at 650 nm (OD_{650}) of these lethally damaged cells did not fall appreciably in saline plus Mg^{2+}.

Conformational changes in C5 LPS. The interaction of Tris with the smooth LPS of C5 was clearly somewhat different than that occurring with Tris plus EDTA. Little LPS was released by Tris, and Tris-induced complement sensitization was readily reversed by Mg^{2+}. With Tris plus EDTA, however, much LPS was liberated from C5, and the complement sensitization induced

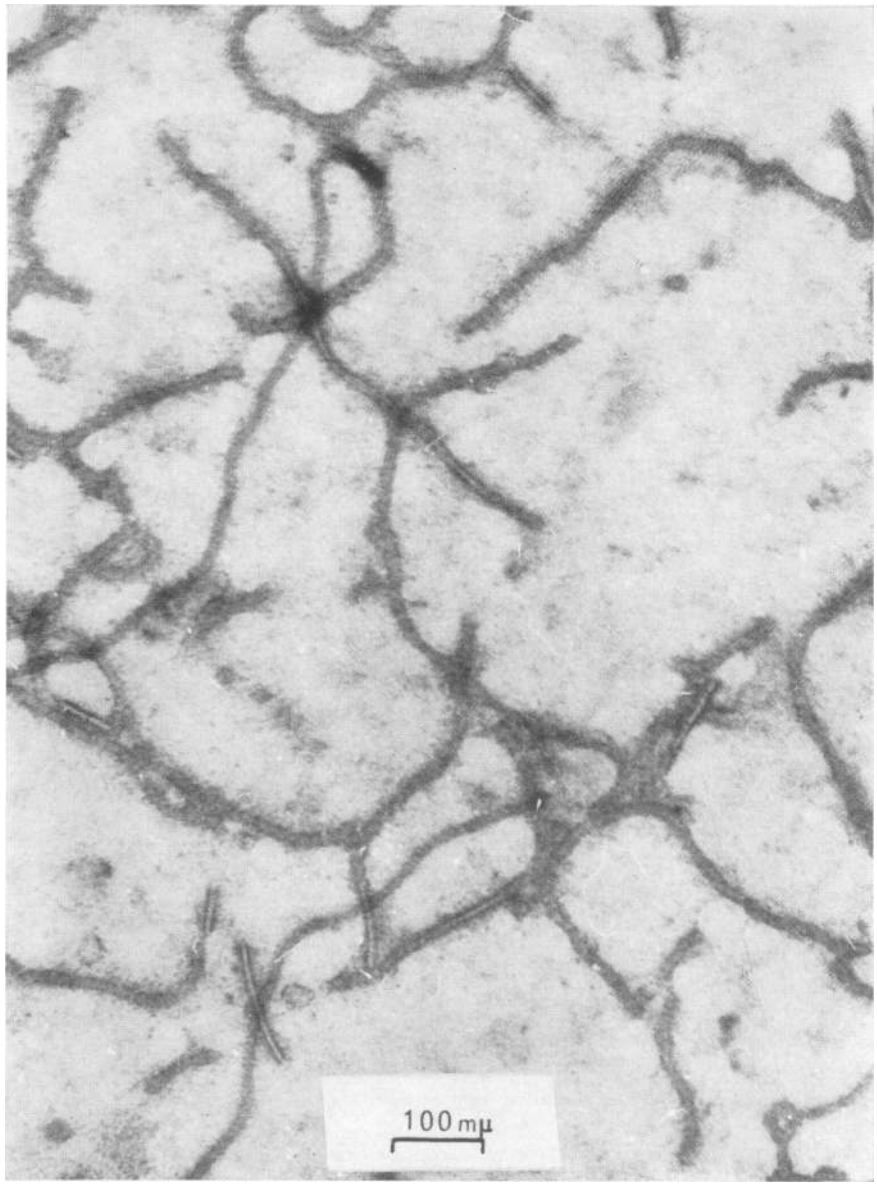

FIG. 2. *LPS in 0.1 M Tris; negatively stained with 2% uranyl acetate.*

could not be reversed by Mg^{2+}. We assumed that the less drastic interaction of Tris alone with C5 LPS produced a relation in the smooth LPS which allowed activated complement components to penetrate and interact with underlying layers of the cell wall. To test this hypothesis, we determined the intrinsic viscosity $[\eta]$ of isolated C5 LPS in several salt solutions. The results are shown in Table 5. The value obtained for $[\eta]$ was low in 0.005 M Mg^{2+} but increased 10-fold in deionized water, 14-fold in either EDTA or Tris-hydrochloride, and 40-fold in Tris plus EDTA. Thus the hydrodynamic volume of C5 LPS increased when Mg^{2+} was absent from the dispersion medium and became larger still in the presence of chelating agents.

Further evidence of conformational changes under these conditions was obtained by microscopic examination. In the electron micrograph (Fig. 1) where the preparation was fixed and stained in the presence of Mg^{2+}, the LPS is contracted to a relatively thick rodlike structure with

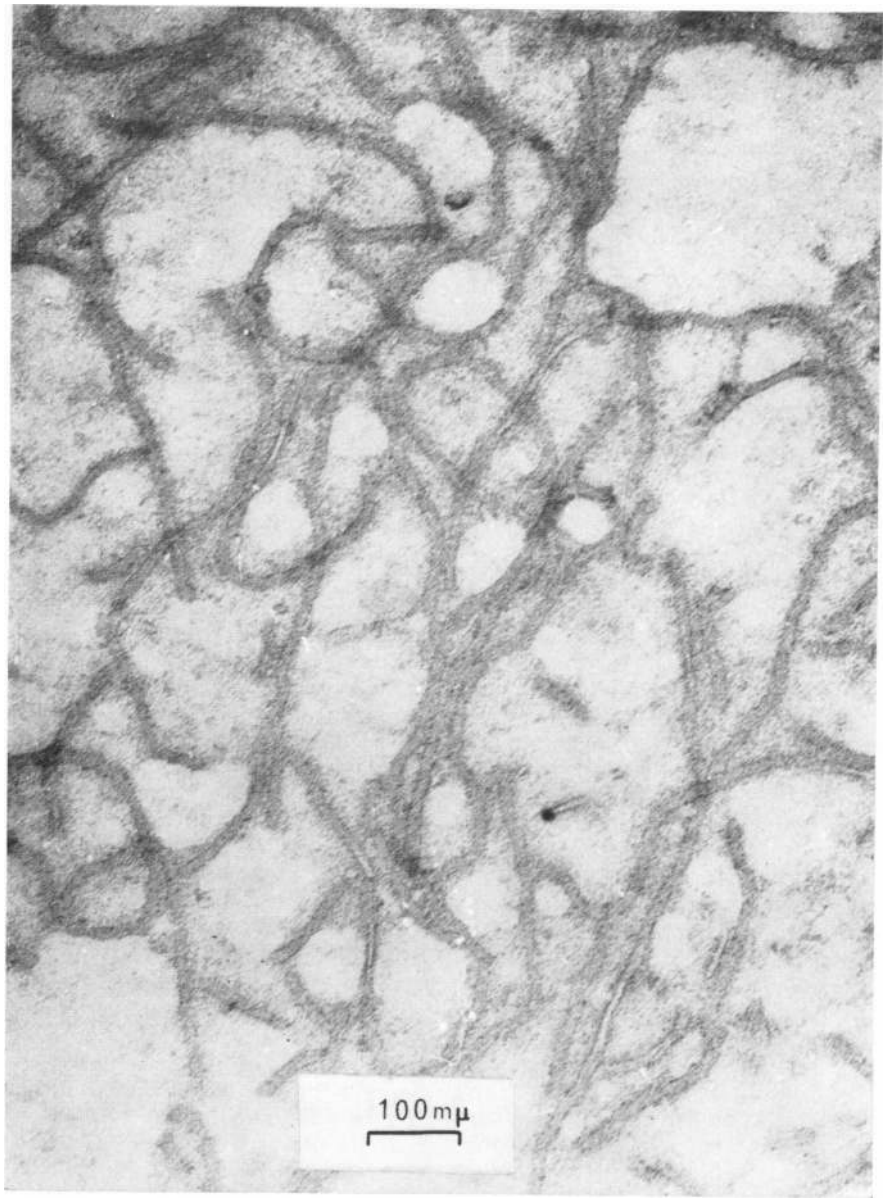

FIG. 3. *LPS in 0.1 M Tris plus EDTA (400 μg/ml); negatively stained with 2% uranyl acetate.*

a diameter of 15.0 to 17.5 nm. Prepared from a dispersion in Tris (Fig. 2) or in Tris plus EDTA (Fig. 3), the LPS had a much more extended form with a diameter of 7.5 to 12.0 nm.

DISCUSSION

These results support the concept that the serum resistance of smooth gram-negative cells is associated with the thick LPS coat which surround them. We have shown that agents which sensitize smooth cells to complement action also produce marked structural changes in the smooth LPS. We suggest that these changes modify the normal barrier function of smooth LPS and permit activated complement components to interact with the underlying cell wall.

We have shown that Mg^{2+} causes marked contraction of free C5 LPS (Table 5 and Fig. 1). We think that this is due to the cross-linking of anionic groups in the lipid A component by the divalent cation. Indeed the precipitation of rough LPS from aqueous solution by Mg^{2+} or Ca^{2+} (6,

14) could well arise in such a manner since divalent cations may produce such a marked contraction of the rough LPS that the lipophilic groups in the lipid A come to occupy the greater part of its surface, thus conferring a strongly hydrophobic character upon the molecule. This possibility is strengthened by the following observations: (i) the insolubility induced in rough LPS by divalent cations is readily reversed by EDTA (6); (ii) univalent Tris ions, which can act as counter ions to anionic groups in lipid A but not cross-link them, cause a marked relaxation of C5 LPS (Table 5); (iii) the relaxation of C5 LPS by Tris is even more pronounced in the presence of EDTA (Table 5).

The univalent Tris cation presumably competes for anionic groups in the LPS of the intact bacterial cell which are normally cross-linked by divalent cations (2, 4, 18) and thereby effects a relaxation in the LPS coat structure. Such changes are not too disruptive since they are readily reversed by Mg^{2+} (Table 2) and do not result in the release of significant amounts of LPS from the cell wall (Table 1). However, in the presence of the strong chelating agent EDTA, Tris ions may largely replace the cross-linking divalent cations, producing a dispersive effect so great as to release large quantities of LPS from the smooth cell wall (Table 1). As a consequence, Mg^{2+} cannot reverse the sensitization which results from this interaction (Table 3) because it is unable to rebind the depleted LPS coat into an effective barrier.

C5-specific antibody complexes formed in the nonsensitizing (saline plus Mg^{2+}) medium, although insensitive to complement when suspended in saline plus Mg^{2+}, were extremely sensitive when washed and resuspended in Tris (Table 2). The sensitization of smooth strains does not, therefore, appear to be due to the fixation of antibody and hence the activation of complement closer to the cell membrane as suggested by Muschel (10) and Reynolds and Rowley (16). Indeed, the sensitization mechanism seems not to act at the level of antibody fixation but rather by promoting some later event, such as the interaction of activated complement components with the cell wall.

Further, it seems unlikely that the sensitization of C5 is primarily due to the fixation of antibodies, other than those directed against O-somatic polysaccharide, to cell antigens which are uncovered by Tris or Tris plus EDTA treatment. BA formed in a nonsensitizing medium (conditions under which internal antigenic determinants would not be exposed) can, after thorough washing, be killed when resuspended in Tris plus complement (Table 2).

The primary lesion produced by the complement system occurs in the lipid moiety of the outer cell wall (3, 5, 7). Consequently, complement damage to bacterial cells may be assessed in terms of the ability of lysozyme to penetrate the outer wall and to degrade the underlying glycopeptide layer. By interacting BA cells, which were specifically labeled in their glycopeptide, with complement plus lysozyme, we have shown (Table 4) that significant release of acid-soluble labeled components occurred only under sensitizing conditions resulting in cell death. In nonsensitizing medium, in which complement did not kill, the lipid moiety of the outer cell wall appeared to suffer little damage as the lysozyme (molecular weight $\sim$ 14,000) was unable to penetrate the cell and reach its glycopeptide substrate. It therefore seems unlikely that smooth gram-negative cells are damaged by complement yet enabled to recover due to the supportive role of their thick LPS coat as suggested by Feingold et al. (5).

The ability of Mg^{2+} to stabilize the bacterial cytoplasmic membrane, which has been observed in other systems (11, 15, 19), was again demonstrated by the observation that the *S. typhimurium* M206 strain which is sensitive to complement in saline plus Mg^{2+}, although killed, did not lyse in this medium since the OD_{650} did not fall appreciably (Table 4).

These investigations generally support the concept that smooth LPS prevents cell damage by preventing activated complement components (C*) from interacting with receptors on the cell wall.

It seems reasonable to assume that at least some C* require to bind to receptors on the cell wall if they are to play their characteristic role in the complement sequence which leads to cell death. The occlusion of such receptors by smooth LPS could therefore abort the complement sequence. Support for this idea comes from observations that smooth gram-negative organisms as well as resisting the bactericidal action of complement also resist phagocytosis (9, 12), an event which is promoted by cell-bound C3 (13, 17).

We propose therefore that the conformational changes induced in smooth C5 LPS by Tris and Tris plus EDTA expose C* receptors, possibly in the lipid core, and thereby sensitize these smooth gram-negative cells to the bactericidal action of complement.

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