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Sensitization of Complement-Resistant Smooth Gram-Negative Bacterial Strains

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Many gram-negative bacteria are susceptible to the bactericidal action of complement in the presence of specific antibody. As a general rule, rough strains are readily killed, whereas smooth strains are relatively resistant. The results of this study show that the serum sensitivity of smooth gram-negative organisms can be controlled by the environmental conditions imposed by the test system. Smooth organisms which are completely resistant to the bactericidal action of immune serum plus complement in the presence of divalent cations become quite sensitive in a test system containing a relatively high concentration of certain univalent cations; this effect is potentiated by ethylenediaminetetraacetic acid. Since it is generally accepted that the serum resistance of smooth gram-negative organisms is associated with the protection afforded by their thick lipopolysaccharide layer, it is suggested that environmental conditions favoring serum sensitization cause some structural disorganization of the smooth lipopolysaccharide of these cells.

Although many gram-negative bacteria are readily killed by serum complement in the presence of specific antibody, rough strains are generally much more sensitive than are smooth strains (6, 17, 25, 26, 27, 31). Indeed, many smooth strains exist which are completely resistant to this bactericidal system when tested in saline (22), saline plus Mg^{2+} (6, 28), phosphate buffer plus Mg^{2+} (26), and acetate-barbiturate buffer (20). Mg^{2+} is frequently added to such test systems, since it was shown to activate complement (16, 23).

The change from the rough to the smooth form of an organism is known to involve significant alteration in the structure and composition of the lipopolysaccharide (15) and was shown to be accompanied by a gradual decrease in sensitivity to the bactericidal reaction (6). It therefore seems likely that the serum resistance exhibited by smooth strains is due to some protective function of their thick lipopolysaccharide layer as previously suggested by Wardlaw (30) and by Muschel (17).

Consequently, the partial removal or structural disorganization of the lipopolysaccharide layer might be expected to weaken the resistance of smooth organisms to the bactericidal action of immune serum. We therefore decided to examine the serum sensitivity of several smooth gram-negative organisms under environmental conditions which we thought might have a disruptive effect on their lipopolysaccharide layer.

Since the combined action of tris(hydroxymethyl)ammonomethane (Tris) and ethylenediaminetetraacetic acid (EDTA) was shown to release considerable quantities of lipopolysaccharide from several gram-negative organisms (14), to uncover murein—the lysozyme substrate which underlies the lipopolysaccharide (24), and to potentiate the antimicrobial action of many substances which are thought to affect membrane permeability, including complement (19), it seemed possible that similar treatments applied to smooth gram-negative strains might break down their resistance to immune serum.

We therefore commenced this study by examining the serum sensitivity of the smooth C5 strain of *Salmonella typhimurium* in assay systems which employed solutions of Tris or Tris plus EDTA as diluents.

MATERIALS AND METHODS

Strains. *S. typhimurium* C5 is the wild-type prototrophic strain virulent for mice described previously by Furness and Rowley (9). *S. typhimurium* M206 is mouse nonvirulent, sensitive to serum, and also sensitive to several rough-specific phages (13). *S. typhimurium* LT2, a smooth strain, was obtained from H. Nikaido, Biochemical Research Laboratory, Massachusetts General Hospital, Boston, Mass. Each of the above strains has the same Kauffman-White O antigens 1, 4, 5, and 12. *S. minnesota* 218S was obtained from O. Luderitz, Max Planck Institut für Immunobiologie, Freiburg, and *S. enteritidis* 795 from Dr. Ribi, Rocky Mountain Laboratory, Mont. Each

is a smooth strain with O antigens 21, 26 and 1, 9, 12, respectively.

Antisera. Antisera were obtained from the rat by injecting intraperitoneally about 10^9 living organisms of either *S. typhimurium* C5, *S. minnesota* 218S, or *S. enteritidis* 795 suspended in physiological saline. The rats were bled from the heart 7 days later, and the serum was pooled and stored in 0.2-ml amounts at −20 C. Antisera were obtained from the rabbit by an initial intramuscular injection of 2×10^8 heat-killed *S. typhimurium* C5 cells in complete Freund's adjuvant, followed by intravenous injections of heat-killed strain C5 cells in physiological saline (injections of 2×10^8 organisms on days 4 and 8 and 10^9 on day 13). Finally, 3 mg of C5 lipopolysaccharide was given intravenously on day 17. The animals were bled from the ear on day 27; the serum was pooled, distributed in 0.2-ml amounts, and stored at −20 C.

Bactericidal assays. Pooled guinea pig serum stored at −20 C was used as the source of complement. To remove specific antibodies, it was adsorbed on the day of use at 0C with approximately 10^{10} living organisms of the strain against which the assay was to be attempted. After centrifugation, the serum was sterilized by passing through a membrane filter (0.45-μm pore size; Gelman Instrument Co., Ann Arbor, Mich.) and was routinely used in the assay system at a final concentration of 1:10. The serum was heated at 56 C for 20 min to inactivate complement when used as a control. The test organisms were those from an exponentially growing culture in Nutrient Broth (Difco) containing about 2×10^8 cells per ml. These cells were washed and resuspended in the solution specified to contain about 5×10^4 cells per ml, and 0.1-ml quantities of this dilution were added to tubes containing 0.9 ml of a mixture of specified salt-solution, complement and antiserum maintained at 37 C. Viable counts were performed by using 0.08-ml samples removed at the time of adding the cells and at suitable intervals thereafter. Viable counts were also performed on organisms added to control tubes containing diluent alone, diluent with specific antibody, diluent with serum, and diluent with heat-inactivated serum and specific antibody.

Under other experimental conditions, test organisms were incubated at 37 C for 30 min in diluent containing specific antibody, washed to remove excess antibody, and redispersed in diluent at 37 C before the addition of serum.

Oxygen uptake. Oxygen uptake was measured by using a YS1 model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). Cells growing exponentially in Nutrient Broth were washed with and suspended in 3 ml of diluent at 37 C to give about 5×10^7 cells per ml; antiserum and complement were then added, and the rate of oxygen consumption was determined.

RESULTS

Immune bactericidal reaction in various salt solutions. The bactericidal activity of antibody and complement was tested against the smooth strain *S. typhimurium* C5 suspended in various salt solutions. The data are presented in Table 1. Although relatively insensitive in phosphate buffer (0.1 M, *p*H 7.0), this strain was somewhat sensitive in physiological saline. However, it showed much greater sensitivity when suspended in Tris-chloride, ethanolamine chloride, or glycine solutions (0.1 M, *p*H 7.0). The addition of 100 μg of EDTA per ml, a concentration which was neither toxic to the test organism nor anticomplementary (Table 2), was observed to increase the serum sensitivity of C5 in Tris-chloride solution, in physiological saline, and in phosphate buffer. The addition of Mg^{2+} (0.002 M) abolished killing in physiological saline and markedly inhibited killing in the Tris-chloride system.

Three other smooth strains of *Salmonella*, *S. typhimurium* LT2, *S. minnesota* 218S, and *S. enteritidis* 795, all completely resistant to the bactericidal action of complement in the presence of specific antibody when suspended in phosphate buffer containing Mg^{2+} (0.002 M), were examined. All three smooth strains were readily killed in the Tris-chloride system. Unlike LT2 and C5, *S minnesota* and *S. enteritidis* did show some sensitivity in physiological saline containing Mg^{2+} (0.002 M). In complete contrast, *S. typhimurium* M206, which has qualitatively the same antigenic

TABLE 1. *Bactericidal action of antibody and complement against Salmonella typhimurium C5 in various salt solutions*[a]

Salt solution at *p*H 7.0	Percentage of inoculum surviving after incubation for 60 min in			
	Salt solution	Salt solution + C′	Salt solution + C′ + Ab	Salt solution + C′ + Ab + Mg^{2+}
0.1 M Tris-chloride	110	210	4	60
0.1 M Tris-chloride + 100 μg of EDTA per ml	120	220	<1	
0.1 M Ethanolamine-chloride	85	250	1	
0.9% Sodium chloride	115	200	75	140
0.9% Sodium chloride + 100 μg of EDTA per ml	110	220	30	
0.1 M Phosphate	170	260	130	150
0.1 M Phosphate + 100 μg of EDTA per ml		200	40	
0.1 M Glycine		95	<1	

[a] Bacteria were incubated at 37 C in salt solution, 1:2,500 rat antiserum (Ab), 1:10 adsorbed guinea pig serum (C′), and Mg^{2+} at final concentration of 0.002 M.

make up as LT2 and C5 but which is probably partly rough (13), was readily killed in all test systems (Table 3).

Inhibition of cell respiration by antibody and complement. The data presented in Fig. 1 were further evidence of the marked sensitivity of *S. typhimurium* C5 to specific antibody and complement when these cells were suspended in Tris-chloride solution. In this system a rapid inhibition of respiration was observed. However, when physiological saline containing 0.002 M Mg^{2+} replaced the Tris-chloride solution as the diluent of the bactericidal system, the rate of oxygen consumption by the cells was found to be only slightly less than that of the control system in which the added serum complement was heat inactivated.

TABLE 2. *Influence of various concentrations of EDTA on the bactericidal action of antibody and complement against Salmonella typhimurium C5*[a]

Concn of EDTA (μg/ml)	Percentage of inoculum surviving after 30 min of incubation in	
	Tris-chloride + Ab + C′	Tris-chloride + Ab + ΔC′
1,000	30	28
500	36	30
250	55	42
100	1	>100
50	2	>100
25	4	>100
10	3	>100
Nil	12	>100

[a] Bacteria were incubated at 37 C in Tris-chloride solution (0.1 M, *p*H 7.5) with 1:2,000 rabbit antiserum (Ab) and 1:10 adsorbed guinea pig serum (C′) or heated guinea pig serum (ΔC′).

TABLE 3. *Bacterial action of antibody and complement against several Salmonella strains in various salt solutions*[a]

Strain	Percentage of inoculum surviving after incubation for 60 min in the presence of specific antibody and complement at *p*H 7.0 in			
	0.1 M Phosphate + 0.002 M Mg^{2+}	Saline + 0.002 M Mg^{2+}	Saline	Tris-chloride
S. typhimurium C5	>100	>100	75	4
S. typhimurium LT2	>100	>100		5
S. minnesota 218S	>100	24		2
S. enteritidis 795	>100	42		4
S. typhimurium M206	8	2	5	<1

[a] Bacteria were incubated at 37 C in each salt solution each containing 1:10 appropriately adsorbed guinea pig serum as a complement source and 1:2,500 rat anti-C5 serum with *S. typhimurium* C5, LT2, and M206; 1:1,000 rat anti-*S. minnesota* 218S serum with *S. minnesota* 218S; and 1:1,000 rat anti-*S. enteritidis* serum with *S. enteritidis*.

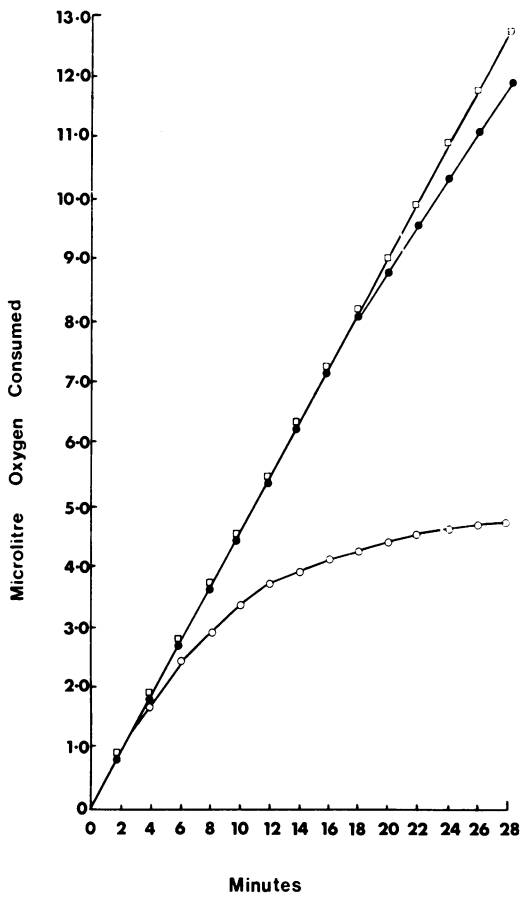

FIG. 1. *Effect of 1:1,000 rat antiserum and 1:10 guinea pig serum on the respiration of S. typhimurium C5. Symbols: ○, suspended in Tris-chloride solution (0.1 M, pH 7.5); ●, suspended in physiological saline containing 0.002 M Mg^{2+}; □, control in which serum was heated to inactivate complement.*

pH and Tris sensitization. Bactericidal assays were carried out, using C5 as the test organism, in Tris-chloride solutions of various *p*H values. As shown in Fig. 2, optimal sensitization was observed at *p*H 7.5. The diminished sensitivity at *p*H 8.0 and 9.0 was not due to inactivation of serum components, as serum incubated at 37 C for 30 min in Tris-chloride at each *p*H level was fully effective when subsequently used in the bactericidal reaction at *p*H 7.0.

Effect of Tris concentration on sensitization. Bactericidal assays were carried out against C5 in

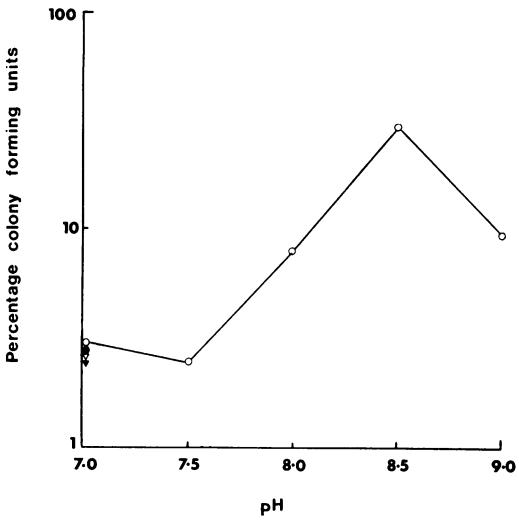

FIG. 2. *Effect of pH on the bactericidal assay versus S. typhimurium C5 suspended in Tris-chloride solution (0.1 M) containing 1:2,500 rabbit antiserum and 1:10 guinea pig serum. Symbols:* ○, *percentage of inoculum surviving 30 min of incubation at the pH levels indicated. The controls* (●, ▽, ▼) *show the percentage of inoculum surviving 30 min of incubation in the bactericidal system, the serum component of which had been previously incubated for 30 min in Tris at pH 8.0, 8.5, and 9.0, respectively. Bacteria incubated in each system in the absence of antiserum increased to between 105 and 115% of the original inoculum during 30 min of incubation.*

concentrations of Tris-chloride solution ranging from 0.20 to 0.01 M at *p*H 7.5. The assay system was kept reasonably isotonic by the addition of suitable amounts of sucrose. Specific antibody was added to each system; after equilibration at 37 C, guinea pig serum was added as a source of complement. The results which are presented in Table 4 showed that C5 is readily sensitized to the bactericidal action of antibody and complement in 0.2 and 0.1 M Tris-chloride solution; however, at concentrations of 0.05 M and below, Tris has diminishing effect.

Increased sensitization by preincubation in Tris. C5 cells were preincubated at 37 C in Tris-chloride solution (0.1 M, *p*H 7.5) for various periods of time, before dilution into a bactericidal system containing serum and specific antibody in Tris-chloride solution, and into a second bactericidal system containing serum and specific antibody in physiological saline. The effect of the period of preincubation in Tris on the survival of cells subsequently diluted into each bactericidal system is shown in Fig. 3. Lengthening periods of preincubation in Tris-chloride solution produced an increase in the initial killing rate in the Tris-chloride bactericidal system. Although the cells were much less suceptible in the test system employing

TABLE 4. *Effect of Tris-chloride concentration on the susceptibility of S. typhimurium C5 to the bactericidal action of antibody and complement*[a]

Diluent		Percentage of inoculum surviving[b]
Tris (M)	Sucrose (M)	
0.2		<1
0.1	0.2	<1
0.05	0.3	9
0.02	0.35	31
0.01	0.38	58
	0.4	94
0.1	0.35	<1

[a] Bacteria were incubated at 37 C in diluent which contained rabbit antiserum (1:2,500) and guinea pig serum (1:10).

[b] After incubation for 45 min in the presence of antibody and complement.

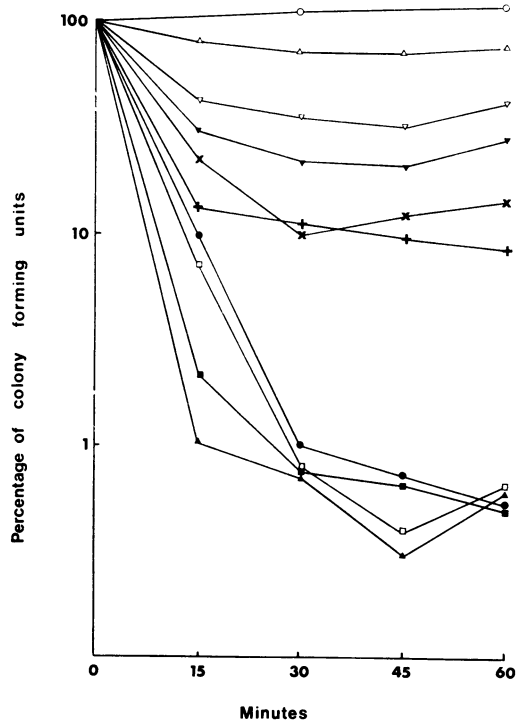

FIG. 3. *Increased sensitivity of S. typhimurium C5 to the bactericidal system after preincubation in Tris-chloride solution. Cells were incubated in Tris-chloride solution (0.1 M, pH 7.5) for 10, 20, 40, or 60 min before dilution into Tris-chloride solution or into physiological saline each containing 1:2,500 rabbit antiserum and 1:10 guinea pig serum. Symbols: diluted into the Tris-chloride bactericidal system after* (●) *10 min,* (□) *20 min,* (■) *40 min, and* (▲) *60 min. Diluted into the physiological saline bactericidal system after* (▽) *10 min,* (▼) *20 min,* (×) *40 min, and* (+) *60 min;* (△) *assay carried out in physiological saline without preincubation in Tris;* (○) *bacteria in Tris alone.*

physiological saline as diluent, the longer they remained in Tris before assay, the more sensitive they became.

Effect of Mg^{2+} or Ca^{2+} on the bactericidal systems. *S. typhimurium* C5 was sensitized to complement action by incubating in Tris-chloride solution containing specific antibody. The cells were subsequently washed, and portions were suspended in Tris-chloride solution containing various concentrations of Mg^{2+} or Ca^{2+}. Serum was then added, and the systems were assayed for bactericidal activity. Figure 4 shows the results of these tests. The bactericidal reaction was progressively inhibited with increasing concentration of either cation; Mg^{2+} appeared to be the more effective inhibitor.

In an attempt to determine whether the inhibitory effect of Mg^{2+} was due to the partial inactivation of complement, we carried out the following experiment. The serum-sensitive strain *S. typhimurium* M206 was presensitized with specific antibody and suspended in physiological saline containing various levels of Mg^{2+}. Portions from each suspension were then incubated with graded

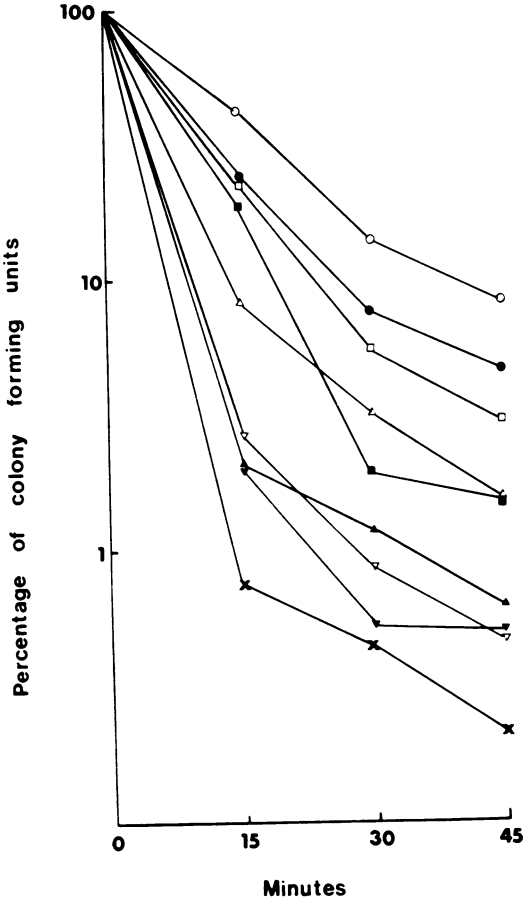

FIG. 4. *Inhibitory effect of various levels of Mg^{2+} and Ca^{2+} on the bactericidal assay of S. typhimurium C5 in Tris-chloride solution (0.1, M, pH 7.5). The cells were incubated in Tris-chloride solution containing 1:1,000 rabbit antiserum for 30 min, washed, and suspended in the appropriate diluent. The assay was performed in the presence of guinea pig serum (1:10). Symbols: ○, 0.008 M Mg^{2+}; □, 0.004 M Mg^{2+}; △, 0.002 M Mg^{2+}; ▽, 0.0005 M Mg^{2+}; ●, 0.008 M Ca^{2+}; ■, 0.004 M Ca^{2+}; ▲, 0.002 M Ca^{2+}; ▼, 0.005 M Ca^{2+}; ×, no divalent cation added.*

TABLE 5. *Effect of various concentrations of Mg^{2+} on the bactericidal action of antibody and complement against Salmonella typhimurium M206*[a]

Molar concn of Mg^{2+} added	Percentage of inoculum surviving 60 min of incubation at a guinea pig serum dilution of		
	1:50	1:75	1:100
Nil	<1	1.5	12
0.0005	<1	<1	2.5
0.001	<1	<1	<1
0.002	<1	<1	1
0.005	<1	1.5	4
0.008	<1	5	22

[a] Bacteria were incubated in saline containing 1:2,000 rabbit antiserum for 30 min, washed, and suspended in physiological saline containing the appropriate level of Mg^{2+} and guinea pig serum.

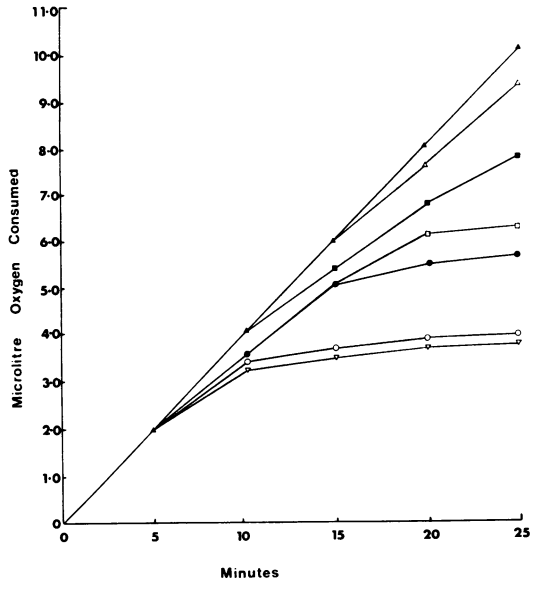

FIG. 5. *Inhibition of respiration of S. typhimurium M206 suspended in physiological saline by 1:2,500 rat antiserum and 1:10 guinea pig serum and the effect when Mg^{2+} is added to the system. Symbols: △, 0.008 M Mg^{2+}; ■, 0.004 M Mg^{2+}; □, 0.001 M Mg^{2+}; ●, 0.0005 M Mg^{2+}; ○, 0.00025 M Mg^{2+}; ▽, no Mg^{2+}; ▲, control system with heated guinea pig serum.*

dilutions of the complement source. Mg^{2+} was observed to potentiate killing at concentrations up to 0.002 M although some inhibition was observed at 0.005 and 0.008 M Mg^{2+} (Table 5). However, with the large excess of complement source (1:10 guinea pig serum) routinely used in our test system, it seems unlikely that these higher concentrations of Mg^{2+} would have any significant inhibitory effects on the bactericidal system by reason of inactivation of the complement system.

We also examined the effect of antibody and complement on the respiration of the serum-sensitive *S. typhimurium* M206 when these cells were suspended in physiological saline containing various concentrations of Mg^{2+} (Fig. 5). Antibody and complement rapidly inhibited the respiration of these cells in physiological saline but this inhibitory effect was antagonized by Mg^{2+}, even at the low concentration of Mg^{2+} which was shown to potentiate the bactericidal action of the complement system (Table 5).

DISCUSSION

The observations reported in this paper demonstrate that certain smooth strains of gram-negative bacteria which were reported to resist the bactericidal action of specific antibody and complement (6, 17, 20, 26) became quite sensitive to this system when the reaction was carried out in Tris-chloride solution. For example, *S. typhimurium* C5, a smooth strain, was completely resistant to the bactericidal system when suspended in phosphate buffer or in physiological saline containing 0.002 M Mg^{2+}. However, when Tris-chloride was used as the diluent in the assay system, C5 was readily killed. The addition of EDTA to the test system, at a concentration which is neither bactericidal nor anticomplementary, not only potentiated the bactericidal effect observed in Tris but also promoted killing in physiological saline and in phosphate buffer.

The effect of antibody and complement on the respiration of C5 cells was also shown to depend on the ionic conditions imposed by the test system. Only a very slight inhibition of oxygen uptake was observed in physiological saline containing Mg^{2+} (0.02 M); however, in Tris, respiration was inhibited almost immediately. This observation demonstrates the rapid onset of the lethal action of immune serum and implies that the cytoplasmic membrane is a point of attack.

As rough gram-negative organisms are usually much more susceptible to the bactericidal action of serum than are their smooth parental strains (6, 17, 26), it seems likely, as suggested by Wardlaw (30), that the resistance of smooth strains is due to the relatively large amounts of lipopolysaccharide associated with their cell wall. For example, the serum-sensitive strain *S. typhimurium* M206 possesses only about half as much lipopolysaccharide as the smooth resistant C5 strain (2). Any protection afforded by the more substantial layer of lipopolysaccharide associated with smooth strains could arise from the shielding effect provided by a thick, compact lipopolysaccharide sheath. Thus, the marked decrease in serum resistance of smooth strains in some salt solutions (Tables 1 and 3) may be due to the effect of certain ions on the structural organization of the lipopolysaccharide layer with the result that this becomes more diffuse and hence less protective.

Much evidence suggests that the structural integrity of lipopolysaccharide is highly dependent on the presence of divalent cations. They have been detected in the lipid A component of the lipopolysaccharide of *Escherichia coli* (4) and are present in the cell wall of *Pseudomonas aeruginosa* (7). The chelating agent EDTA solubilizes lipopolysaccharide (12) and liberates lipopolysaccharide from *E. coli* (14). It was proposed (3, 5) that lipopolysaccharide may be composed of subunits cross-linked via divalent cations and that the cross-links occur between phosphate groups in the lipopolysaccharide. Univalent cations may be expected to compete with divalent cations for negatively charged groups in the cell wall, and in the presence of a chelating agent the divalent cations would be largely replaced by the univalent cations present as previously suggested by Vos (29). Indeed this mechanism may also explain the very considerable contribution made by Tris to the complexing action of EDTA in several instances (3, 10, 11, 24). The replacement of cross-linking divalent cations by univalent cations unable to form cross-links would significantly alter the structural organization of the lipopolysaccharide and would be most likely to modify its cell protective function. Our observations that *S. typhimurium* C5 is slightly serum sensitive when suspended in physiological saline and becomes more sensitive when EDTA is added to the system are compatible with this suggestion. A similar role has been proposed for Na^{+} when EDTA in hypertonic saline was used to remove divalent cations from the wall of *P. aeruginosa* (3).

Since Tris has some chelating activity (1) and is also a source of univalent cations, we propose that it too is able to disrupt lipopolysaccharide by replacing divalent cations with the univalent Tris ion and thus sensitize smooth gram-negative bacteria to the bactericidal action of immune serum. Our data showing that C5 cells become less resistant to immune serum the longer they are preincubated in Tris (Fig. 3) and that at Tris concentrations below 0.1 M the degree of sensitization

decreases (Table 4) support this concept, as one woud expect such an exchange process to be time and concentration dependent. We would argue that the increased serum senstivity of C5 in Tris plus EDTA (Tables 1 and 2) and its decreased sensitivity in Tris plus divalent cations (Table 1 and Fig. 4) further support this concept of ion replacement, since the extent to which Tris ions could replace the divalent cations associated with anionic groups in the lipopolysaccharide would be inversely related to the concentration of divalent cations in the system.

Although the inhibition of C5 killing systems by Mg^{2+} could possibly be due to the partial inactivation of complement at the higher Mg^{2+} levels, this is certainly not so at lower levels of Mg^{2+}, which clearly activate the complement system (Table 5). The effect of Mg^{2+} in our bactericidal systems using the two stains of *S. typhimurium* C5 and M206 appears to depend on the nature of the lipopolysaccharide of the test organism. C5, the strain with the more substantial lipopolysaccharide coat, is protected by the same low levels of Mg^{2+} which make the M206 strain more sensitive. It appears, therefore, that Mg^{2+} has two distinct effects in the immune bactericidal system. It can potentiate the bactericidal action against normally sensitive strains by activation of the complement system but it can also protect smooth strains from the lethal effects of immune serum by stabilizing their outer lipopolysaccharide coat.

The inhibitory effects of relatively high concentrations of Mg^{2+} on complement-mediated hemolysis (16) and on immune bacteriolysis (17, 18) may well be due to the stabilization of the membranes of the test cells rather than to a direct inhibitory effect on the complement system. Our observations (Fig. 5) suggest that the cytoplasmic membranes of test cells may be stabilized by Mg^{2+} as this cation was observed to antagonize the inhibitory effect of immune serum on the respiratory process in *S. typhimurium* M206. The loss of permeability control by these cells was delayed even at those levels of Mg^{2+} which were shown to activate complement in the bactericidal reaction (Table 5). It seems likely therefore that as previously suggested by Muschel and Jackson (18), Mg^{2+} is able to stabilize the cytoplasmic membrane, probably by preventing the release of lipid residues (32) formed by complement action.

By observing the effect of *p*H on the survival of *S. typhimurium* C5 in a bactericidal system containing 0.1 M Tris-chloride solution as diluent, we were able to obtain some evidence that the Tris cation is involved in sensitization. Since Tris is a relatively weak base, its degree of dissocation falls from above 0.9 at *p*H 7.0 to about 0.1 at *p*H 9.0. Consequently, the decrease in serum bactericidal activity observed in Tris at higher *p*H values suggests the involvement of the Tris ion in sensitization. Our controls showed that complement was not inactivated at the higher *p*H levels, and complement activity is fully expressed at *p*H 8.5 in Tris buffer since Wardlaw (30) obtained optimal killing of the rough strain *E. coli* Lilly with immune serum under these same conditions. We did observe a greater susceptibility of cells in Tris at *p*H 9.0 than at *p*H 8.5 but this may well be due to the effect of alkalinity per se on the cell walls (21). We were unable to follow this line of investigation with ethanolamine, the ionization of which would be much less affected by *p*H since it is a strong base, as this substance was toxic to the test organisms above *p*H 8.0.

Since ethanolamine yields univalent cations and glycine dipolar ions, either of which is able to act as a counter ion to negatively charged groups in the cell wall, we assume that both ethanolamine and glycine sensitize smooth gram-negative organisms to the bactericidal action of serum by acting in a similar manner to that which we proposed for Tris. This mechanism could also explain the conversion of *E. coli* 0117 to serum sensitivity by diphenylamine (8), although its action is probably more specific.

Whether these organic ions cause breaches in the outer cell wall through the release of lipopolysaccharide, as in the case of the EDTA plus Tris system described by Lieve et al. (14), or whether they simply cause the lipopolysaccharide layer to become more diffuse and hence more permeable to the effector substances produced when complement is activated is the subject of further investigation.

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