JOURNAL OF BACTERIOLOGY, Dec. 1968, p. 2118-2126

Vol. 96, No. 6
Printed in U.S.A.

Locus of the Action of Serum and the Role of Lysozyme in the Serum Bactericidal Reaction

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Received for publication 23 September 1968

The mechanism of the lethal action of human serum on a rough strain of *Escherichia coli* was investigated by use of serum with and without lysozyme, in medium of low and high osmotic pressure, with cells radioactively labeled in the peptidoglycan polymer, and by electron microscopy. The results suggested that there are two separate components in the bacterial cell wall that afford structural support for the cell. Lysozyme attacked one of these, the peptidoglycan polymer. Serum damaged the other, which is probably the peripherally located lipopolysaccharide-phospholipid complex. The cell wall damage caused by lysozyme-free serum promptly resulted in cell death under usual conditions. In plasmolyzed cells, however, the wall damage was not lethal, presumably because the membrane of the plasmolyzed cell was protected from secondary lethal changes which otherwise occur.

Serum kills many gram-negative organisms by a reaction requiring antibody against a cell envelope antigen, at least some of the components of the complement system, and possibly other serum factors (20). Since the discovery of this phenomenon 80 years ago (7), much has been learned about it, but several important questions remain unanswered or incompletely answered. What is the nature of the lethal event? What serum factors are required for killing? How do serum-resistant gram-negative organisms differ from serum-sensitive ones? What is the role of this immunological system as a host defense against gram-negative infections? With these questions in mind, our studies of the serum bactericidal reaction were initiated. The locus of the bactericidal action of serum on a rough strain of *Escherichia coli* is the subject of this and the following paper (12); the studies focus on the primary lesion caused by the serum factors and the secondary events that result in death of the bacterial cell.

Under the conditions usually employed in studies of the serum bactericidal reaction, the killed bacteria show evidence of both cell wall and cell membrane damage (24, 25). The sequence of events leading to this end result has not been well defined. The results of our experiments indicate that the primary, complement-mediated effect of serum is on the nonpeptidoglycan portion of the bacterial cell wall. This wall damage is not necessarily a lethal event unless lysozyme degrades the peptidoglycan of the wall, or unless secondary damage, presumably to the cell membrance, occurs. These conclusions are based on studies of morphological changes during the reaction as seen by electron microscopy, on studies of the effect of hypertonic medium on the serum bactericidal reaction, on studies of the degradation of specifically labeled peptidoglycan polymer, and, as reported in the following paper, on studies of changes in the compartmentation of enzymes during the serum bactericidal reaction.

MATERIALS AND METHODS

Organisms and cultural conditions. E. coli 200P, a K-12 strain obtained from Boris Magasanik, requires leucine, threonine, and vitamin B_1 and is cryptic for β-galactosidase ($i^+ z^+ y^-$). Stock cultures were transferred on nutrient agar slants at monthly intervals and stored at 4 C. Organisms were grown in dextrose phosphate broth (Albimi Laboratories, Inc., Flushing, N.Y.) from a small inoculum to stationary phase on a rotary shaker at 37 C; the cells were washed three times before use with 0.05 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (*p*H 7.2) containing 10^{-3} M Mg^{++}.

A diaminopimelic acid (DAP)- and lysine-requiring auxotroph (*E. coli* 200P DAP^- Lys^-) was selected from *E. coli* 200P by use of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co. Inc., Milwaukee, Wis.) and the technique of Adelberg et al. (1). Tritiated DAP, with a specific activity of 190 mc/mmole, was purchased from Nuclear-Chicago Corp. (Des Plaines, Ill.). Before use, it was further

purified by thin-layer chromatography on Silica Gel G (Brinkman Instruments, Inc., Westburg, N.Y.) in an *n*-butyl alcohol–pyridine–water–acetic acid (60:40:30:3) solvent system, and was eluted from the gel with broth. After unlabeled DAP (Nutritional Biochemicals Corp., Cleveland, Ohio) was added to give a final concentration of 15 mg/ml with a specific activity of 7 mc/mmole, the broth was inoculated and the organisms were grown for 18 hr. The stationary-phase cells were washed six times with cold medium before use. To check the specificity of the labeling, the radioactive cells were hydrolyzed for 18 hr in 6 N HCl at 110 C; the HCl was removed by repeated evaporation under reduced pressure. A sample of the hydrolysate was chromatographed in descending fashion on Whatman number 1 paper in both *n*-butyl alcohol–acetic acid–water (63:10:27) and methanol-water-10 N HCl-pyridine (80:17.5:2.5:10) solvent systems. In both solvent systems, over 95% of the label was present in the area of authentic *meso*- or DD-DAP.

Complement sources and antisera. Serum used as the complement source was a pool from several healthy donors. Immediately after collection and multiple absorptions in the cold with heat-killed (80 C for 60 min), saline-washed *E. coli* 200P, the serum was frozen at −70 C in small portions until use. The absorptions lowered but did not completely remove antibody against the organisms; at dilutions of the absorbed serum greater than 1:5, the addition of specific antiserum or purified antibody was required to effect killing of the bacteria; unabsorbed serum was lethal at greater than a 1:100 dilution without added antiserum.

Lysozyme removal from serum was accomplished by two 15-min absorptions with 3 mg of washed bentonite (Fisher Scientific Co., Pittsburgh, Pa.) per ml at 4 C. The complement hemolytic activity of the serum was lowered by less than 5% by the bentonite absorption. Unlike the observations of Glynn and Milne (13), addition of 20 μg of lysozyme (Worthington Biochemical Corp.) per ml to the bentonite-absorbed serum restored the bactericidal activity to normal.

Antiserum to *E. coli* 200P was prepared with rabbits by use of heat-killed, washed organisms. After three weekly subcutaneous injections of 10^{10} organisms, the rabbits were bled 5 days after the last injection. Such antiserum had detectable activity in the bactericidal reaction at a 10^{-4} dilution.

Bactericidal reaction. Washed bacteria were suspended in test medium, and the pooled, human serum was added as indicated, usually to a final concentration of 10% (v/v). In all experiments, antiserum was present at a final dilution of 10^{-3}. After incubation at 37 C for the times indicated, samples were diluted appropriately and plated on nutrient agar to determine the number of viable organisms remaining. Controls with heat-inactivated serum (56 C for 30 min) were always run in parallel and the per cent killing was calculated.

Microscopy. Organisms for election microscopy were fixed in 3% glutaraldehyde for at least 2 hr at 4 C, postfixed for 1 hr in 2% OsO_4, dehydrated in alcohols, embedded in Epon resin 812 (Fisher Scientific Co.), and sectioned on a Porter-Blum MT-2 ultramicrotome (Ivan Sorvall Inc., Norwalk, Conn.); the sections were picked upon Formvar-coated grids, poststained with uranyl acetate and lead citrate, and examined with a Philips EM 100 microscope at an accelerating voltage of 60 kv.

RESULTS

Morphological appearance of serum-killed E. coli. The bacterial cell wall of *E. coli* 200P as it usually appears, a wavy, amorphous structure with little discernible detail, is shown in Fig. 1. Figures 2 to 4 show the structural changes that accompany the lethal action of whole serum (Fig. 2) and lysozyme-free serum (Fig. 3 and 4).

In whole serum, greater than 99% of the cells are killed and converted from their rod shape into spheroplasts. The residual cell wall assumes a layered structure and is seen as an electron-lucent layer sandwiched by two electron-dense layers; the cell membrane appears to be internal to the wall structure (Fig. 2). As described by Davis et al. (9), lysozyme-free serum results in the formation of killed rods. Figure 3 shows clearly that the cell wall complex in this situation consists of three rather than two electron-dense layers, as observed previously by Spitznagel in *E. coli* 0117 (24).

Lysozyme, therefore, results in degradation of a component of the cell wall that is responsible, at least in part, for the rod shape of this organism. Morphologically, this component appears as an electron-dense layer by electron microscopy. This structure is undoubtedly the peptidoglycan polymer of the cell and most likely is the innermost electron-dense layer of the three layers that comprise the cell wall (10, 18).

Also of note is the formation of spherules of "membrane" material (Fig. 4) which regularly appear in association with killing by lysozyme-free serum. These spherules appear as globules in negatively stained preparations and as membranous circles or layers in section. They seem to orginate from the cell wall and appear similar to the lipopolysaccharide spherules observed by Work et al. in lysine-starved *E. coli* (16). Thus, even in the absence of lysozyme, serum leads to a loss of cell wall material from the cell and to a change in the morphological appearance of the cell wall.

Plasmolysis and the protective effect of hypertonic medium on the serum bactericidal reaction. In an attempt to dissociate the effect of serum on the cell wall from that on the cell membrane, the bactericidal reaction was performed in hypertonic sucrose (0.6 M). In this concentration of a poorly penetrable solute such as sucrose, water

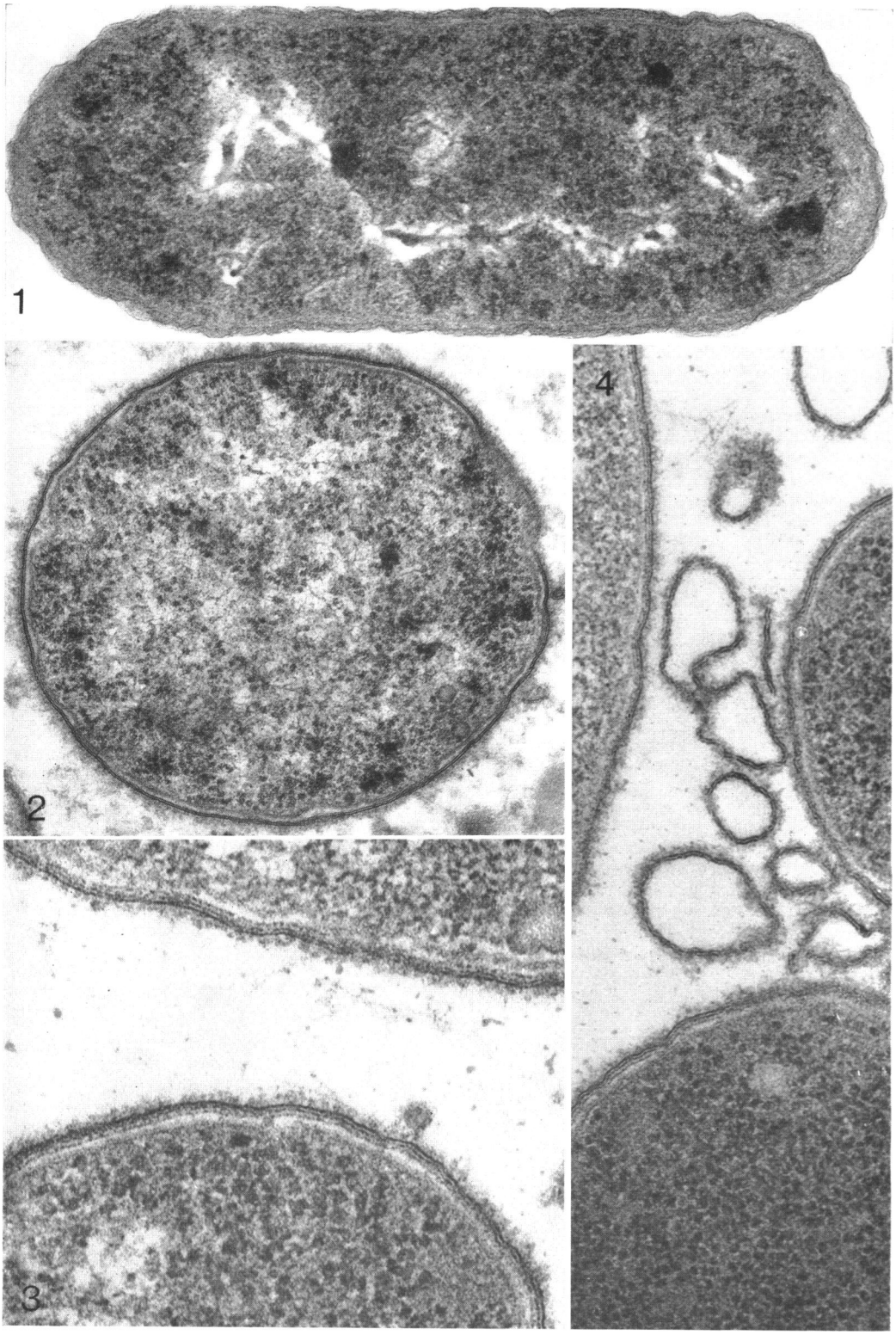

FIG. 1–4

flows out from the cell and the cell membrane contracts within the rigid cell wall (19). This is called plasmolysis, and it is not lethal for the organism. Typical plasmolyzed *E. coli* 200P cells are shown in Fig. 5. When the action of serum is examined with and without 0.6 M sucrose in the reaction mixture, a striking difference between serum with and without lysozyme is revealed. Under these experimental conditions, whole serum kills regardless of the presence of sucrose, whereas the lethal effect of lysozyme-free serum is inhibited by 0.6 M sucrose (Table 1).

Early in our studies of this phenomenon, we observed that cells that had been grown overnight in 0.6 M sucrose were fully sensitive to serum with or without lysozyme, unlike cells that were put into the sucrose-containing medium only shortly before challenge with serum. A representative experiment illustrating this point is shown in Table 2. This finding by itself is strong evidence that the protective effect of sucrose does not result from inhibition of the complement system, but rather from an effect of the sucrose on the bacteria; this protective effect of sucrose is abolished when the bacteria are grown for a few generations in the sugar.

Thus, it appears that sucrose per se does not inhibit the action of serum on the organisms, but in the absence of lysozyme this action of serum is not lethal for plasmolyzed bacteria. Lysozyme by itself does not kill *E. coli* under these conditions. Even in sucrose, however, the serum-mediated damage and the lysozyme-induced peptidoglycan degradation result in irreversible disorientation of the cell wall structure; i.e., spheroplast formation. Hence, these observations suggest that the serum-mediated lesion involves the bacterial cell wall and that, in the absence of lysozyme, plasmolysis prevents subsequent lethal changes from occurring.

Plasmolysis can be reversed by dilution of the medium to lower the osmotic pressure. Also, since sucrose enters the cell slowly by a respiratory-linked process (17), plasmolysis gradually decays in the presence of serum or other suitable energy sources. This reversal of plasmolysis does not occur at 4 C or in the absence of an energy source. Similarly, although hypertonic sucrose markedly delays the bactericidal action of lysozyme-free serum, the inhibition gradually decays. To define further the relation between plasmolysis and the bactericidal effect of serum, the kinetics of the lethal action of lysozyme-free serum in 0.6 M sucrose were correlated with the state of plasmolysis of *E. coli* (Table 3). The degree of plasmolysis, rated as 0 to 3^+, was determined by electron microscopy on samples removed and fixed at the times indicated. Figure 6 shows the same plasmolyzed cells shown in Fig. 5, 90 min after addition of heat-inactivated serum; the bacteria show no evidence of plasmolysis and were graded 0, whereas the cells in Fig. 5 were graded 3^+. In the experiment presented in Table 3, the delayed lethal action of the serum in sucrose is clear as is the temporal association of the lethal event with the reversal of plasmolysis. It is tempting to suggest that the process of plasmolysis confers resistance upon the bacteria to the lethal action of lysozyme-free serum.

Figure 7 shows *E. coli* 200P cells, from the experiment presented in Table 2, that were grown in the presence of 0.6 M sucrose, washed, resuspended in 0.6 M sucrose, and then sampled for electron microscopy before the addition of serum. These cells show only minimal plasmolysis (1^+) and are sensitive to the serum bactericidal reaction, again confirming the inverse relationship between plasmolysis and serum sensitivity.

Role of peptidoglycan in the bactericidal reaction. The peptidoglycan polymer of the cell wall was radioactively labeled with ^{3}H-DAP, as described above, in order to follow the fate of this structure during the bactericidal reaction and to further evaluate its role in cell morphology. After the labeled cells were treated as shown in Table 4, the percentage of the total DAP released into the medium was calculated by determining the radioactivity in the cell suspension versus that in the supernatant fluid after centrifugation. Several interesting results emerge on correlation of these values with viability and cell morphology. When spheroplasting takes place, either with whole serum or with ethylenediaminetetraacetic acid (EDTA) and lysozyme, well over half of the label is released into the medium. Once spheroplasting occurs, the bacteria are no longer viable, at least on conventional media. Lysozyme-free serum, although lethal for the bacteria, produces little release of radioactivity. Under

FIG. 1. *Control E. coli 200P. Little structure can be discerned in the cell wall.* × *93,000.*

FIG. 2. *Representative cell 30 min after exposure to 10% whole serum. These cells assumed a spherical shape. Two electron-dense layers in the cell wall are clearly visible.* × *56,000.*

FIG. 3. *Cells treated with lysozyme-free serum for 30 min. The cells remained rod shaped but were not viable. The cell walls have three electron-dense layers.* × *110,000.*

FIG. 4. *Same preparation as in Fig. 3 showing numerous blebs or vesicles that were present. They were bounded by two electron-dense layers.* × *130,000.*

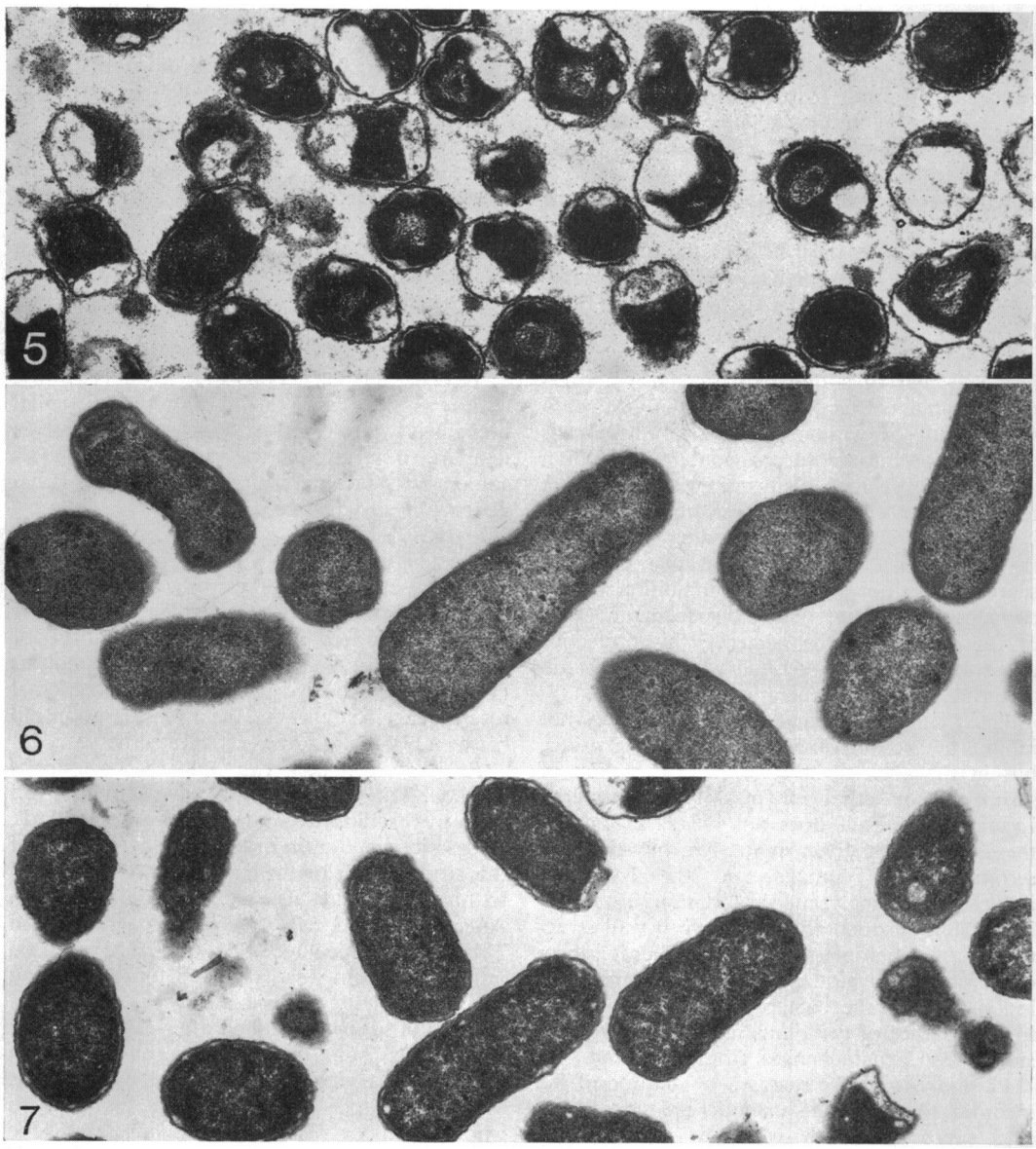

FIG. 5. *E. coli 200P showing extensive plasmolysis occurring soon after the cells were suspended in 0.05 M Tris (pH 7.2) and 10% heat-inactivated serum with 0.6 M sucrose.* × *12,000.*

FIG. 6. *Same culture as above after 90 min of incubation at 37 C. Complete reversal of plasmolysis occurred.* × *27,000.*

FIG. 7. *E. coli 200P fixed soon after cells were suspended in Tris buffer with 0.6 M sucrose. They differed from cells in Fig. 5 in that they were grown in the presence of 0.6 M sucrose. Only minimal plasmolysis occurred.* × *27,000.*

these conditions, lysozyme, which by itself has no effect on the viability or the rod shape of the bacteria, often produces greater than 50% release of label and, hence, degradation of peptidoglycan polymer.

It should be noted that measurements of radioactivity released into the supernatant fluid may significantly underestimate the degree of depolymerization of the peptidoglycan polymer or the functional importance of the lysozyme action.

TABLE 1. *Effect of hypertonic sucrose on the serum bactericidal reaction*

Reaction medium[a]	Per cent killing[b]	
	Whole serum[c]	Lysozyme-free serum[c]
Buffer-Mg^{++}	>99	>98
Buffer-Mg^{++}-sucrose	>99	0

[a] *E. coli* 200P were suspended at approximately 10^3 bacteria/ml in either 0.05 M Tris-chloride buffer (*p*H 7.2) containing 10^{-3} M $MgCl_2$ or in the same solution with sucrose added to give a final concentration of 0.6 M.

[b] Thirty minutes after the addition of serum to a final concentration of 10% (v/v), samples were taken to determine the number of viable organisms remaining.

[c] Heating serum to 56 C for 30 min abolished the lethal effect.

TABLE 2. *Effect of growth of the bacteria in hypertonic sucrose on the serum bactericidal reaction*

Sucrose in growth medium	Sucrose in test medium	Per cent killing by lysozyme-free serum
M	*M*	
0	0	>99
0	0.6	0
0.6	0	>94
0.6	0.6	>94

[a] Except for the different growth conditions of *E. coli* 200P, the experimental design was the same as that in Table 1.

Subunits of the polymer may remain bound to some other wall structure and not be released into the medium in soluble form. Furthermore, hydrolysis of a relatively small percentage of the *N*-acetyl glucosamine linkages may abolish the effective structural supportive role of the peptidoglycan polymer.

It would follow that if the peptidoglycan is susceptible to lysozyme alone, pretreatment of the cells with lysozyme followed by exposure to lysozyme-free serum should be lethal and should result in spheroplast formation either in the absence of presence of 0.6 M sucrose. Accordingly, cells were exposed to 50 μg of lysozyme per ml for 60 min at room temperature, washed three times with buffer, and then treated with lysozyme-free serum. The serum was lethal and resulted in spheroplast formation (Table 5). Heat-inactivated serum was without effect in all cases in the presence or absence of 0.6 M sucrose. These results and the results of the experiments with ^{3}H-DAP suggest that the peptidoglycan is degraded during the lysozyme preincubation, and the subsequent serum-mediated wall damage then results in spheroplasting. An alternative explanation for the results is that lysozyme molecules remain adherent to the cell through the washing pro-

TABLE 3. *Plasmolysis and the serum bactericidal reaction*

Time	Per cent killing by lysozyme-free serum[a]		
	No sucrose (10% serum)[b]	0.6 M Sucrose	
		10% Heat-inactivated serum	10% Serum[b]
min			
0	0	0 (3^+)[c, d]	0 (3^+)[c]
15	69		0 (3^+)
30	99		0 (2^+)
45	99		0 (2^+)
60	99		39 (1^+)
75			79 (1^+)
90		0 (0)[e]	90 (0)

[a] *E. coli* 200P suspended at approximately 10^8 bacteria/ml in 0.05 M Tris-chloride (*p*H 7.2) containing 10^{-3} M $MgCl_2$ and sucrose where indicated. Samples (0.1 ml) were taken at the times indicated and were diluted appropriately for determination of viable numbers.

[b] Final serum concentration (v/v).

[c] The numbers in parentheses represent the degree of plasmolysis. Samples (3 ml) were used for electron microscopy.

[d] *See* Fig. 5.

[e] *See* Fig. 6.

TABLE 4. *Effect of EDTA, lysozyme, and serum on the bactericidal reaction, cell morphology, and peptidoglycan degradation*

Addition[a]	Per cent killing	Cell morphology[b]	Per cent of total radioactivity released from cells[c]
Nothing	0	Rods	8.9
Lysozyme (50 μg/ml)	0	Rods	58
EDTA (2×10^{-3} M)-lysozyme (50 μg/ml)	99	Spheroplasts	84
Whole serum (10%)	99	Spheroplasts	90
Lysozyme-free serum (10%)	99	Rods	24
Heat-inactivated serum (10%)	0	Rods	24

[a] Additions were made to suspensions of washed, labeled *E. coli* 200P DAP$^-$lys$^-$ (approximately 10^8 bacteria/ml) in 0.05 M Tris-chloride buffer (*p*H 7.2) and were incubated for 30 min at 37 C.

[b] As determined by phase-microscopic observations.

[c] Calculated as per cent of total cellular radioactivity found in the supernatant fluid after treatment and centrifugation at 30,000 × *g* for 15 min.

TABLE 5. *Effect of lysozyme preincubation on the serum bactericidal reaction*

Lysozyme preincubation[a]	Sucrose in test medium on incubation with serum[b]	Per cent killing[c]	Cell morphology
0	0	88	100% Rods
0	+	18	100% Rods
+	0	99	100% Spheroplasts
+	+	99	100% Spheroplasts

[a] *E. coli* 200P was incubated at 23 C with lysozyme in 0.05 M Tris-chloride buffer (*p*H 7.2), and the cells were washed four times by centrifugation with buffer. The cells were then resuspended at about 10^8 bacteria/ml before the addition of serum.

[b] Sucrose (0.6 M); 10% lysozyme-free serum.

[c] After incubation for 30 min at 37 C.

[d] As determined by phase-microscopic observation.

cedure and function after the addition of the serum. Eagon and Carson, however, using rhodamine-labeled lysozyme, have shown that no fluorescent material remains bound to *Pseudomonas* organisms after washing the cells with water or saline (11).

In Table 6, the results of an experiment are presented in which the bacteria were exposed to lysozyme-free serum, collected by centrifugation, and resuspended in the initial medium containing lysozyme but not serum. The *E. coli* cells that were protected from the bactericidal reaction by sucrose were killed by the subsequent addition of lysozyme alone, whereas control cells were much less affected. In addition, many of the bacteria pretreated with lysozyme-free serum were subsequently converted into spheroplasts after exposure to the lysozyme. The experiments tabulated in Tables 5 and 6 suggest that sequential damage to the two supporting structures of the cell wall is lethal, whereas damage to one or the other can be tolerated under certain conditions.

DISCUSSION

The cell walls of *Enterobacteriaceae* are complex structures consisting of several components. Peptidoglycan, which is responsible for the rigidity of gram-positive bacteria, appears to share this responsibility with more peripheral wall components in gram-negative bacteria (8, 26). With bacteria labeled specifically with ^{3}H-DAP, a label that is restricted to the peptidoglycan polymer, extensive degradation caused by lysozyme is observed with no effect on the rod shape of the organisms. Spheroplast formation occurs only after addition of the lysozyme-free serum which further damages the cell wall structures. On the other hand, the wall damage caused by lysozyme-free serum alone does not result in spheroplast formation unless lysozyme is added previously, simultaneously, or subsequently.

The supportive role of these two components of the bacterial cell wall probably varies in different organisms and in the same organism under different conditions. EDTA, which affects the functional integrity of the lipopolysaccharide (LPS) while having no known action on peptidoglycan, causes spheroplasting or lysis of some *Pseudomonas* (27) organisms but not of most strains of *E. coli*. On the other hand, lysozyme alone does not affect the rod shape of stationary-phase *E. coli* but at times causes spheroplast formation from growing organisms (4) which apparently have defects at areas of new cell wall synthesis.

The identity of the more peripheral component of the cell wall important for structure is not definite. However, the evidence strongly points to an LPS-phospholipid complex. Work et al. have shown that LPS is associated with large amounts of phosphatidylethanolamine (PE); when they are overproduced during conditions of unbalanced growth, the complex is excreted into the medium and assumes the form of spherules or layers of materials with a "unit membrane" structure (15). Rothfield found that in vitro LPS associates with PE resulting in similar membrane layers or spherules (21). It appears likely that the two outer dense layers of the cell wall represent this LPS-PE complex. The recent demonstration (22) that phospholipases weaken the walls of gram-negative organisms strongly

TABLE 6. *Effect of lysozyme on E. coli 200P after treatment with lysozyme-free serum*[a]

Serum incubation[b]	Lysozyme[c]	Per cent killing	Morphology[d]
	μg/ml		
Lysozyme-free serum	50	97	38% Spheroplasts, 62% rods
	0	39	100% Rods
Inactivated lysozyme-free serum[e]	50	4	100% Rods
	0	20	100% Rods

[a] Cells suspended at about 10^8 bacteria/ml in 0.05 M Tris buffer (*p*H 7.2) containing 10^{-3} M Mg^{++} and 0.6 M sucrose.

[b] Incubated for 30 min at 37 C with 10% serum.

[c] Cells suspended in original medium minus serum and incubated at 23 C for 60 min.

[d] A total of 100 cells counted by phase-contrast microscopy.

[e] Incubated at 56 C for 30 min.

implicates this complex as an important structural component in the bacterial cell wall.

Experiments with red blood cells indicate that phospholipids may be the substrates for the action of the terminal components of the complement system, resulting in the release of free fatty acids (23). Since PE is an important component of the gram-negative bacterial cell wall in association with the LPS, the LPS-PE complex appears to be a likely target of the complement-mediated wall damage. The observation that membranous blebs are released from *E. coli* cells whose walls are damaged by lysozyme-free serum is consistent with this; these blebs are morphologically similar to those identified by Knox et al. (15) as the LPS-PE complex. In association with this bleb formation, a definite lamellar pattern can be discerned in the wall of the lysozyme-free serum-treated cells; this pattern replaces the wavy wall structure seen in the untreated cells. One explanation for this is that the LPS-PE complex is usually a redundant structure and release into the medium yields a tighter envelope in which the lamellar structure can be seen.

This study confirms that cell wall damage caused by serum occurs in the absence of lysozyme. It is clear that this damage is not necessarily lethal if one creates conditions, such as plasmolysis, to protect bacteria from the lethal secondary changes that usually occur. By elegant electron microscopic techniques, Bayer has demonstrated that, under the usual conditions in which the cytoplasm of the bacterial cell is hypertonic with respect to the medium, the cell membrane exerts pressure on the wall, and weakening of the wall by a variety of techniques results in protrusion of the cell membrane through the wall and the subsequent loss of viability (2, 3). If this type of passive secondary membrane damage, following the primary action of complement on the cell wall, is responsible for the lethal action of the lysozyme-free serum, then the reason for the protective effect of hypertonic medium becomes clear. Evidence in support of this hypothesis is presented in the following paper.

By use of electron microscopy, Bladen et al. (5, 6) have shown what may actually be physical holes or pits in the bacterial surface and in isolated LPS after treatment with serum. We have seen similar "holes," but at the present time one must be cautious about interpreting such a phenomenon, as pointed out by Humphrey et al. (14). Even if they are actual holes, they may only represent superficial holes in layers of lipid adsorbed from the complement source onto the cell surface.

ACKNOWLEDGMENTS

We thank Klara Rev for careful technical assistance.

This investigation was supported by Public Health Service grant AI-06313 from the National Institute of Allergy and Infectious Diseases. D. S. F. was the recipient of Public Health Service Research Career Development Award 5 KO3 AI 35455 from the National Institute of Allergy and Infectious Diseases.

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