JOURNAL OF BACTERIOLOGY, Dec. 1968, p. 2127-2131
Vol. 96, No. 6

Locus of the Lethal Event in the Serum Bactericidal Reaction[1]

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Received for publication 23 September 1968

Hypertonic sucrose inhibited the bactericidal activity of lysozyme-free serum against a rough strain of *Escherichia coli*. The duration of the inhibition correlated with the duration of plasmolysis caused by the sucrose. Although the lethal action of the serum was delayed, the prompt release of alkaline phosphatase by the cells suggested that nonlethal damage to the cell wall had taken place under these conditions. In contrast, the crypticity of the cells for β-galactosidase did not deteriorate until the viability of the bacteria began to decrease. It is concluded that the primary site of action of serum is at the bacterial cell wall; however, in the absence of lysozyme, the lethal event was subsequent damage to the bacterial cell membrane.

In the preceding paper (6), evidence was presented that the primary lesion of *Escherichia coli* caused by serum is in some component of the bacterial cell wall, most likely the lipopolysaccharide (LPS)-phospholipid complex. Although it was shown that this damage is not necessarily lethal, serum-treated bacteria are effectively killed if the peptidoglycan polymer, which offers the remaining structural support for the cell after damage to the outer wall layer, is degraded by lysozyme yielding spheroplasts, or, if, in the absence of lysozyme, secondary damage occurs resulting in nonviable cells which retain their rod shape. Further evidence, included in this paper, implicates the membrane as the locus of these secondary, lethal changes.

In this paper, we describe experiments that show that bacterial cell membrane damage follows complement-mediated cell wall damage and that the membrane damage parallels the loss of viability of the bacteria in the absence of lysozyme. The interpretation of these experiments depends on the apparent compartmentation of enzymes in *E. coli*. Various degradative enzymes, such as alkaline phosphatase (EC 3.1.3.1), seem to exist in large part at a superficial location in the bacterium, possibly external to the cell membrane or in what has been called the periplasmic space (8, 14). Hence, the release of alkaline phosphatase to the medium is thought to reflect disruption of the integrity of the bacterial cell wall, but not necessarily of the cytoplasmic membrane. β-Galactosidase (EC 3.2.1.23) represents a class of enzymes that is found only inside the cell; i.e., in the area delimited by the cell membrane. The entry into the cell of substrates of β-galactosidase which are normally excluded must reflect damage to the cell membrane.

E. coli 200P, a mutant cryptic for β-galactosidase (i.e., lacking a transport system for β-galactosides but capable of synthesizing the enzyme), was exposed to lysozyme-free serum under various conditions including those that allow cell wall damage to occur but inhibit killing (6). The release of alkaline phosphatase into the medium and the entry of β-galactosides into the cells were measured; we conclude that the alkaline phosphatase release parallels the action of the complement system on the cell wall and that the loss of the permeability barrier to the usually excluded β-galactosides parallels death of the cells.

MATERIALS AND METHODS

Organisms and cultural conditions. E. coli 200P (6) is inducible for β-galactosidase but lacks a transport system for β-galactosides ($i^+ z^+ y^-$). The enzyme alkaline phosphatase is also inducible under conditions of phosphate deprivation. The organisms were grown from a small inoculum to stationary phase at 37 C on a rotary shaker with the phosphate-limited medium of Garen and Levinthal (7), which is a tris(hydroxymethyl)aminomethane (Tris)-buffered minimal salts medium; isopropyl-β-D-thiogalacto-pyranoside at 5×10^{-4} M was added to the medium. The cells were washed three times with Tris-chloride buffer (*p*H 7.2) before use.

[1] Presented in part at the 7th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Ill., 25-27 October 1967.

Complement sources and antisera. These were prepared as previously described (6).

Bactericidal reaction. The washed organisms were suspended at a concentration varying from 5×10^7 to 10^8/ml in 0.05 M Tris buffer (*p*H 7.2) with 10^{-3} M Mg^{++} and 0.6 M sucrose where indicated. A final 10% concentration of bentonite-absorbed serum was used in all cases. At the times indicated, samples were taken in triplicate and diluted appropriately to determine, by plate count on nutrient agar, the number of viable organisms remaining. Samples (1 ml) were removed and filtered through 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.); the filtrate was saved for assay of alkaline phosphatase activity. Additional samples (3 ml) were taken and centrifuged at 10,000 $\times$ *g* for 10 min; the supernatant fluid was decanted, and the cells were immediately assayed for evidence of β-galactosidase activity.

Assays. For the assay of alkaline phosphatase activity, *p*-nitrophenyl phosphate disodium tetrahydrate (no. 104 phosphatase substrate Sigma Chemical Co., St. Louis, Mo.) was prepared at 0.2 mg/ml in 1 M Tris-chloride buffer (*p*H 8.0). To 1.8 ml of this substrate, 0.2 ml of culture filtrate was added; this mixture was incubated at 23 C for 30 min, and the optical density (OD) at 410 nm was determined.

For assay of β-galactosidase activity, *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma Chemical Co.) was used as the substrate at 4 mg/ml in 0.2 M $NaPO_4$ buffer (*p*H 7.0). The cells from the bactericidal reaction mixture were resuspended in 3.0 ml of 0.05 M Tris-chloride buffer (*p*H 7.0) with 10^{-3} M Mg^{++}. ONPG solution (0.5 ml) was added and incubated at 23 C for 30 min; 1.5 ml of 1 M Na_2CO_3 was added to stop the reaction, and the OD at 410 nm was determined.

RESULTS

Figure 1 demonstrates the delayed lethal effect of lysozyme-free serum on *E. coli* 200P when the reaction was run in 0.6 M sucrose. In the experiments presented, the kinetics of the release of alkaline phosphatase into the medium and the change in the entry of β-galactosides into the cells were measured after treating cells with lysozyme-free serum in the absence and presence of 0.6 M sucrose.

Figure 2 correlates the lethal effect of the serum, the release of alkaline phosphatase into the medium, and the accessibility of substrates of β-galactosidase to the enzyme. Since measurement of both enzyme activities is based on the liberation of the *o*-nitrophenyl chromagen, both of the enzyme activities are plotted as the OD at 410 nm generated by a sample of supernatant fluid in one case and by a cell sample in the other, in the standard assays described above. Although the cell concentrations differed somewhat in the experiments and the total enzyme concentrations in the induced cells varied slightly, the amount of alkaline phosphatase released into the medium varied between 20 and 40% of the total enzyme content in all of the experiments reported, as measured after sonic treatment of the organisms for 10 min in a Raytheon 10-kc sonicator.

In Fig. 2, all three parameters measured began to change simultaneously and continued to move in similar directions throughout the course of the experiment. Heat-inactivated serum was without effect. From this experiment, one cannot dissociate temporally cell wall damage, cell membrane damage, or the lethal event.

A quite different result is seen in the experiment presented in Fig. 3. The organisms were suspended in 0.6 M sucrose 5 min before the addition of lysozyme-free serum. During the 60 min of this experiment, no killing was observed but alkaline phosphatase was released into the medium; no release was observed with serum heated to 56 C for 30 min. Figure 4 represents an experiment done under the same conditions; however, this experiment was carried out longer and the cells at each point were also examined for their ability to exclude β-galactosides. The release of alkaline phosphatase clearly preceded the lethal event, whereas the change in β-galactoside permeability was delayed and paralleled killing.

β-Galactosidase activity was not found in the medium in any of the experiments in which lysozyme-free serum was used. However, when whole serum was used, large amounts of the enzyme were released into the supernatant fluid.

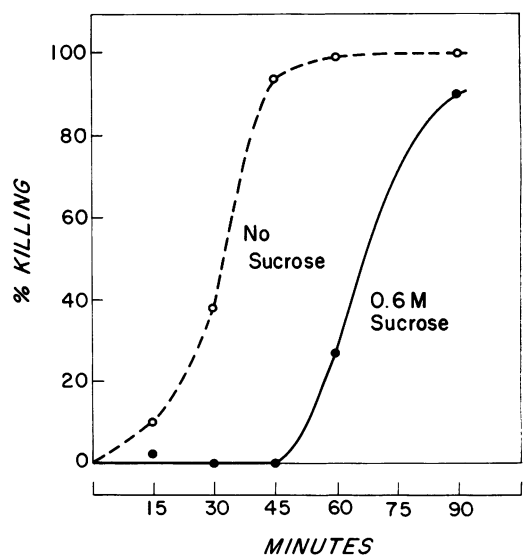

FIG. 1. *Kinetics of the lethal effect of 10% lysozyme-free serum on E. coli 200P in buffer with and without 0.6 M sucrose.*

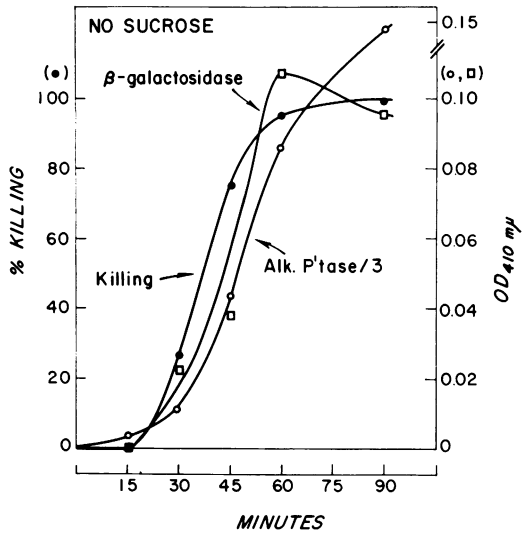

FIG. 2. *Kinetics of the effect of 10% lysozyme-free serum in buffer on viability, release of alkaline phosphatase to the medium, and β-galactosidase activity of the cells.*

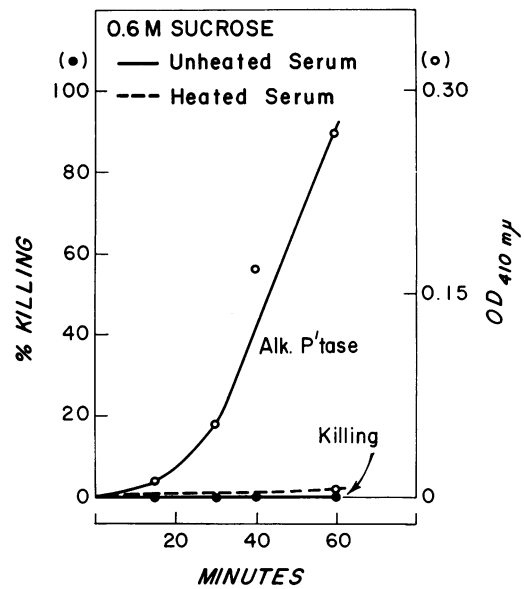

FIG. 3. *Kinetics of the effect of 10% lysozyme-free serum in buffer containing 0.6 M sucrose on viability and alkaline phosphatase release into the medium.*

DISCUSSION

The entry of β-galactosides into organisms cryptic for β-galactosidase is a well-accepted indication of cell membrane damage. Several lines of evidence suggest that alkaline phosphatase release from the cells reflects cell wall damage. The enzyme is almost quantitatively released into the medium with spheroplast formation induced by ethylenediaminetetraacetic acid (EDTA) and lysozyme (10), and also with plasmolysis in sucrose-EDTA followed by rapid osmotic shock (14–16). Furthermore, by histochemical techniques, alkaline phosphatase has been located between the outer layers of the cell wall and the cytoplasmic membrane (5). Finally, mutant *E. coli* with defective cell walls, necessitating osmotic support, lose alkaline phosphatase into the medium (11); in gram-positive organisms thought to have more porous cell walls, alkaline phosphatase is regularly found as an exoenzyme (3). That the complement system causes the release of alkaline phosphatase into the medium strongly suggests that the restraining barrier for alkaline phosphatase, the cell wall complex, has become defective as the result of the action of complement.

The observations reported in this and the preceding paper (6) suggest the following sequence of events during the serum bactericidal reaction. The complement system acts in a still unspecified manner on the LPS-phospholipid complex of the bacterial cell wall, resulting in loss of wall material from the cell and in an impairment of the supportive function of this structure. However, with the peptidoglycan polymer intact, the cells usually remain rod-shaped. When lysozyme is present, the cell often loses its rod structure, becomes a spheroplast, and is not viable on conventional media. The cell is rapidly killed

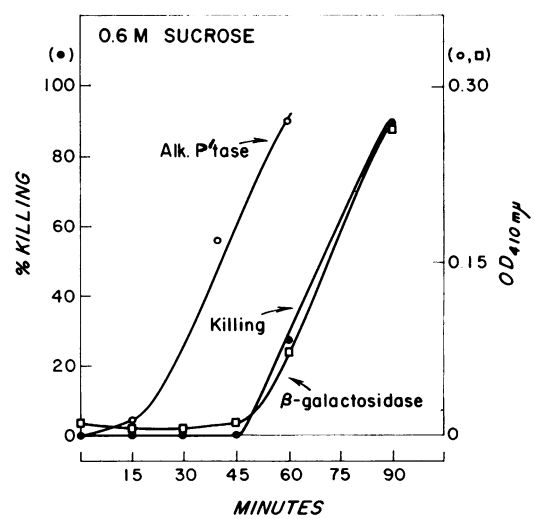

FIG. 4. *Kinetics of the effect of 10% lysozyme-free serum in buffer containing 0.6 M sucrose on viability, release of alkaline phosphatase into the medium, and β-galactosidase activity of the cells.*

even in the absence of lysozyme, and the loss in viability correlates with evidence of damage to the cell membrane.

The cause of the proposed lethal membrane damage is not clear. Possibly, the complement system also directly damages the bacterial cell membrane as suggested by Muschel (13); this would mean that two separate complement-mediated reactions occur during the course of bacterial killing. Although there is no direct evidence on this point, it seems more likely that the membrane damage occurs as a consequence of the usually existing osmotic forces which become lethal in the presence of a defective cell wall. This concept is strongly supported by the fact that contraction of the cell membrane, plasmolysis, caused by hypertonic sucrose inhibits the bactericidal reaction more than it inhibits the cell wall damage (6). Bayer (1) recently demonstrated that, even in extensively plasmolyzed cells, several hundred areas of contact remain between the bacterial cell wall and the cell membrane. These areas of contact are places at which one might expect complement-mediated membrane damage to occur, even in plasmolyzed cells, if the complement system does cause the membrane damage. Since no membrane damage can be detected in the plasmolyzed cells, this further supports the importance of the osmotic forces to the lethal effect.

This formulation of the events in the serum bactericidal reaction would explain several previous observations. (i) Gram-positive organisms are resistant to killing by serum. These organisms do not contain the LPS-phospholipid complex which appears to be the substrate for the primary action of the complement system in the serum bactericidal reaction. (ii) Growing *E. coli* are more sensitive to the lethal action of serum than stationary-phase organisms. It has been estimated that growing *E. coli* have an internal osmotic pressure several times higher than that in nongrowing organisms (2). If the osmotic forces are responsible for killing, then one reason for the differential sensitivity is clear. Furthermore, growing cells normally have defects in their cell walls at areas of new wall synthesis and, perforce, more active autolytic enzyme systems which would also result in less stable bacteria. The events occurring during the serum bactericidal reaction could be dissociated with stationary-phase but not with rapidly growing bacteria. (iii) L colonies can be grown from organisms "killed" by whole serum, although no viable units of any kind remain when lysozyme-free serum is used (9). L colonies consist of organisms (L forms or spheroplasts) with defective cell walls which no longer adequately support the organisms under usual conditions; the cell membranes are apparently intact and, although cell division is difficult in the absence of a rigid cell wall, it does occur in the supportive medium, resulting in the L colonies. Hence, spheroplasts induced by whole serum do not appear to have damaged membranes. At first this seems paradoxical. However, if the rigid cell wall is rapidly converted to a distensible structure by serum in the presence of lysozyme, then the internal osmotic pressure can be accommodated by expansion of the sphere with no membrane damage.

It should be noted that these experiments were performed on *E. coli* K-12, a rough organism. In general, this type of organism is significantly more sensitive to serum than smooth *E. coli* (12, 17). In fact, many smooth organisms are resistant to the lethal action of the antibody-complement system. Smooth organisms differ from the rough ones in that they contain increased amounts of O-antigen polysaccharide in the LPS of the cell wall. Chedid and co-workers (4) and Rowley (18) have suggested that the target antigen of the bactericidal antibody is not O-antigen, as had previously been suggested, but rather "rough antigen" internal to the O-polysaccharide and protected by it in smooth organisms. Some smooth organisms are serum-sensitive; such organisms may have an O-antigen surface which incompletely blocks the antigen important in the serum bactericidal reaction. There are two alternative explanations for the serum-resistance of many smooth organisms: (i) these organisms may suffer complement-mediated wall damage but not be killed, since the more substantial cell wall continues to perform its supportive function; or (ii) since smooth organisms contain O-antigen at distal sites (19), complement fixation may occur too far from the lipid-containing substrate to cause wall damage.

ACKNOWLEDGMENTS

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 Career Development Award 5 KO3 AI 35455 from the National Institute of Allergy and Infectious Diseases.

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