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STUDIES ON THE IMMUNE BACTERIOLYSIS

XIII. LEAKAGE OF ENZYMES FROM *ESCHERICHIA COLI* DURING IMMUNE BACTERIOLYSIS[1]

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SUMMARY *Escherichia coli* B and its mutants were converted to BAC′ cells by lysozyme-free antiserum and lysozyme-free complement (RL). BAC′ cells had the normal morphological rod shape, and lost little β-D-galactosidase, an intracellular enzyme.

With lysozyme BAC′ cells were converted to serum spheroplasts and lost β-D-galactosidase. This liberation of the enzyme was inhibited in hypertonic medium. In contrast, plakin, a phospholipase A from rabbit blood platelets, converted BAC′ cells to non-stainable rod-shaped ghosts, and liberated β-D-galactosidase even in hypertonic sucrose medium.

Alkaline phosphatase, a surface-bound enzyme, was released from bacteria during the formation of BAC′. The release was accelerated by addition of either lysozyme or plakin.

The results obtained were discussed in relation to the localization of these enzymes and to the mode of their liberation during immune bacteriolysis.

INTRODUCTION

It was discovered in this laboratory that lysozyme activity is essential in immune bacteriolysis, i.e., formation of serum spheroplasts, on addition of antibody and complement (INOUE *et al.*, 1959). However, lysozyme alone cannot attack gram-negative bacteria except under certain conditions such as in the presence of ethylenediamine tetraacetate (EDTA). It is unknown how lysozyme can reach its substrate which is deeply embedded in the cell wall when immune bacteriolysis proceeds.

This paper reports the liberation of bacterial

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enzymes using lysozyme-free antiserum, lysozyme-free complement serum and the extraneous enzymes, egg white lysozyme and plakin, a phospholipase A from blood platelets (HIGASHI *et al.*, 1966).

From the results obtained it is postulated that antibody and complement, even without any participation of lysozyme, make "channels" in the cell wall, through which enzyme proteins pass in or out, resulting in the access of extraneous enzymes to their substrates. With lysozyme, this leads to the destruction of the rigid mucopeptide structure of the cell wall, and formation of spheroplasts. With plakin, when enzyme reaches its substrate on the cytoplasmic membrane, ghosts are formed, even in hypertonic medium.

MATERIALS AND METHODS

1. *Bacteria*

Escherichia coli B, Hershey strain was obtained by courtesy of Dr. Matsushiro, Department of Microbial Genetics, the Research Institute for Microbial Diseases, Osaka University. The streptomycin-resistant mutant, strain 004, was obtained by selection of spontaneous mutants. The alkaline phosphatase-constitutive mutant, strain $004P^+$ 5 was obtained from strain 004 by treatment with N-methyl-N'-nitro-N-nitrosoguanidine by the method of ADELBERG *et al.* (1965).

2. *Growth media*

a) *Y medium* containing 20 g of polypeptone, 5 g of NaCl, 1 g of yeast extract and 5 g of Na_2HPO_4 in 1,000 ml of distilled water, pH 7.2, was used for maintaining bacterial strains, and for solid medium 1.5% agar was added.

b) *ρ-medium* containing 5.0 g of NaCl, 1.0 g of $(NH_4)_2PO_4$, 1.0 g of K_2HPO_4 and 0.2 g of $MgSO_4 \cdot 7H_2O$ in 900 ml of distilled water, pH 7.2, was autoclaved. Nine parts of ρ-medium were combined with 1 part of 5% lactose solution which was sterilized separately before use, and use for growth of bacteria in which β-D-galactosidase was induced (Lac-ρ-medium).

c) *Tris medium* was prepared as described by ECHOLS *et al.* (1961).

3. *Diluent*

Isotonic phosphate-buffered saline containing M/200 phosphate buffer, pH 7.3, 0.1% gelatin, 0.0005 M $MgCl_2$ and 0.00015 M $CaCl_2$ was used as diluent (GPB-saline).

Twelve % sucrose in GPB-saline was used as a hypertonic diluent. To adjust the tonicity of reaction mixtures, 50% sucrose in GPB-saline was added.

4. *Antiserum*

Rabbit antiserum against *E. coli* B strain Hershey killed by heating at 56°C for 60 min, was inactivated at 56°C for 60 min, and then treated with bentonite (Wako Pure Chemicals, Ltd.) at a concentration of 10 mg/ml in an ice bath for 10 min. After incubation the bentonite was removed by centrifugation. This procedure was repeated 3 times. The resultant antiserum was free from lysozyme activity. The antiserum gave an agglutination titer of 1 : 12,800 against the homologous strain and its mutants, and was stored at −20°C without addition of any preservative.

5. *Lysozyme-free complement (RL):*

Fresh guinea pig serum was treated as described in the previous paper to remove lysozyme activity (INOUE *et al.*, 1959). In this paper numbers in parenthesis following RL represents the number of repetitions of the bentonite treatment. RL was stored in a deep-freezer of −70°C.

6. *Lysozyme*

Crystalline egg white lysozyme was obtained from Sigma Chemical Co., St. Louis, Mo. The activity of this enzyme was estimated by the method of SHINKA *et al.* (1962).

7. *Plakin*

Rabbit plakin was obtained by the method of HIGASHI *et al.* (1963).

8. *Assay of β-D-galactosidase*

Two ml of test sample were mixed with 1.0 ml of 5.0×10^{-4} M *o*-nitrophenol-β-D-galactoside in M/50 phosphate buffer, pH 7.5. After incubation at 37°C for 20 min, 2.0 ml of 1 M Na_2CO_3 were added. The optical density was measured at 420 mμ. For the titration of total intracellular enzyme, bacteria were lyzed by incubation with 25 μg/ml lysozyme M/1,000 ethylenediamine tetraacetate (EDTA), at pH

7.2 at 37°C for 90 min, and after centrifugation the supernatant was used for titration.

9. *Assay of alkaline phosphatase*

One ml of test sample was mixed with 3.0 ml of 3.0×10^{-3} M *p*-nitrophenyl phosphate in 1 M Tris HCl buffer, pH 8.0. The mixture was incubated at 37°C for 30 min and then 1.0 ml of 12% K_2HPO_4 was added. The optical density was measured at 420 mμ.

10. *Degree of morphological change*

4+ shows that all the bacteria were converted to serum spheroplasts. −shows no spheroplast formation. ★★★★ shows that all the bacteria were converted to nonstainable ghost-like cells.

RESULTS

1. *Leakage of β-D-galactosidase during immune bacteriolysis*

In the previous paper (AMANO *et al.*, 1956b) it was shown that intracellular β-D-galactosidase leaks out from *E. coli* B cells as immune bacteriolysis proceeds. Hypertonic medium, obtained by addition of sucrose, prevents the leakage of the enzyme, while the morphological change to spheroplasts proceeds in hypertonic medium in parallel with that in ordinary isotonic medium. It was also demonstrated that, in addition to antibody and complement, lysozyme is essential for formation of serum spheroplasts by immune bacteriolysis. Bacteria treated with lysozyme-free antiserum and lysozyme-free complement (RL), i.e., BAC′ cells, have the normal rod shape and are not susceptible to the lytic effect of 0.05% sodium deoxycholate, which destroys serum spheroplasts resulting in the release of intracelullar nucleic acid into the medium (INOUE *et al.*, 1959).

The leakage of β-D-galactosidase was investigated using lysozyme-free antiserum and RL.

E. coli B/SM, strain 004 was grown in Lac-ρ- medium at 37°C overnight with mechanical shaking. The bacteria were harvested by centrifugation in the cold, and were washed twice with cold GPB-saline and resuspended in the same buffer at a concentration of 1.0×10^9 cells/ml. At time 0, the suspension was pipetted into flasks containing four volumes of immune-bacteriolytic reagents in GPB-saline. The concentrations of the reagents after the addition of the bacteria were: 1/500 lysozyme-free antiserum, 1/150 RL (3) and 5.0 μg/ml egg white lysozyme (flask A). Flasks B and C contained no lysozyme, flask C received heat-inactivated RL instead of active RL. At intervals, 2.5 ml aliquots were taken and immediately centrifuged in the cold. Release of β-D-galactosidase into the supernatant was measured. As controls, spontaneously liberated enzyme from untreated bacteria (bacteria only control) and the total amount of enzyme (100% enzyme control) were measured as mentioned in Materials and Methods. Morphological changes in stained specimen were examined microscopically.

As shown in Fig. 1, in the presence of excess

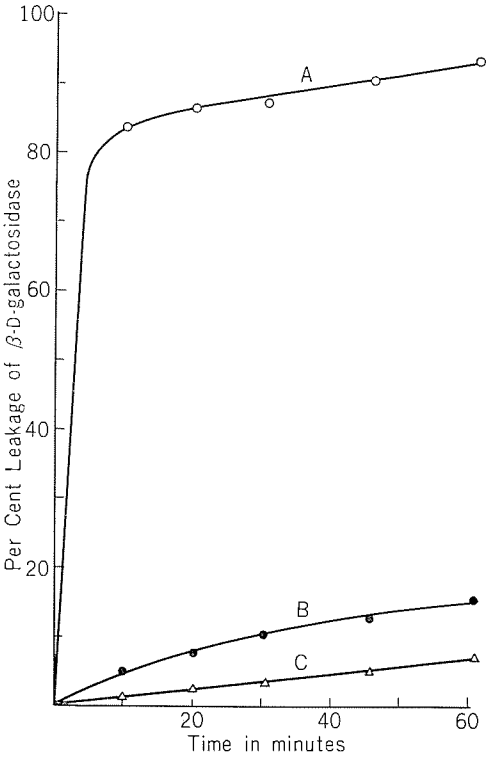

FIGURE 1 Leakage of β-D-galactosidase during immune bacteriolysis. A: in the presence of 5.0 μg lysozyme/ml; B: lysozyme-free (RL) system; C: heat-inactivated RL control. Morphological changes are shown in Table 1.

TABLE 1 *Morphological changes during immune bacteriolysis* (Fig. 1)

min	10	20	30	60
A	2+	3+	4+	4+
B	—	—	—	—
C	—	—	—	—

For explanation see text.

lysozyme, the intracellular enzyme, β-D-galactosidase, leaked out very rapidly. In the absence of added lysozyme, the enzyme also leaked out slightly and no serum spheroplasts were formed.

2. *Leakage of β-D-galactosidase into the medium from antibody-and-complement-treated bacteria (BAC′)by plakin or lysozyme*

As reported in the previous paper, lysozyme can react with washed BAC′, resulting in their conversion to serum spheroplasts, while bacteria which are treated with lysozyme and then washed, cannot be coverted to spheroplasts by the action of antibody and RL (INOUE *et al.*, 1959). Lysozyme is known to act on the polysaccharide backbone of the mucopeptide complex (murein bag) of bacterial cell walls. In gram-negative bacteria the mucopeptide complex is embedded in the cell wall and is not susceptible to lysozyme unless it becomes exposed. This happens under certain conditions, such as in the presence of EDTA. Plakin, on the other hand, is a phospholipase A which can attack the protoplast membrane of *Bacillus megaterium* (HIGASHI *et al.*, 1966). Usually plakin alone cannot act on gram-negative bacteria, but it makes them nonstainable like protoplast ghosts when it is added to an immune-bacteriolytic system (AMANO *et al.*, 1953).

E. coli B/SM, strain 004 grown in Lac-ρ-medium was harvested and washed as in the previous experiment. The bacteria were divided to two portions. One portion was washed once more with GPB-saline. Then the bacteria were converted to BAC′ by incubating them with 1/500 lysozyme-free antiserum and 1/150 RL (3) in GPB-saline at a concentration of 2.0×10^8 bacteria/ml at 37°C for 60 min. After incubation, the bacteria were centrifuged in the cold, washed twice with cold GPB-saline and resuspended in the same buffer.

The BAC′ suspension at a concentration of 1.0×10^9 bacteria/ml in GPB-saline was poured into a flask containing four volumes of a mixture of egg-white lysozyme and rabbit plakin in GPB-saline (A). The final concentrations of enzyme in the reaction mixture were 5.0 μg of lysozyme/ml and 1/3 plakin. Systems without plakin (lysozyme alone: B), without lysozyme (plakin alone: C) and without both (D) were also set up.

At intervals a 2.5 ml aliquot was taken from each flask and centrifuged immediately in the cold. The amount of β-D-galactosidase in the supernatant was measured. As controls, the supernatant from the BAC′ cells at time, 0 and 100% intracellular enzyme were measured. The morphological changes of BAC′ were examined microscopically.

As shown in Fig. 2, the intracellular enzyme, β-D-galactosidase, leaked out into the surrounding medium by the action of either lysozyme or plakin. The synergistic effects of

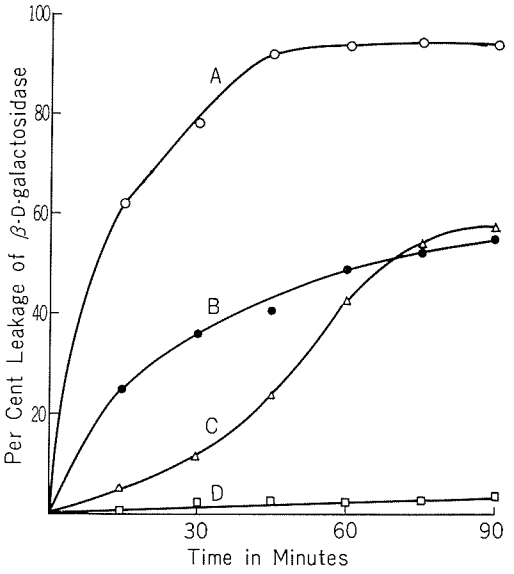

FIGURE 2 Leakage of β-D-galactosidase from BAC′ cells in isotonic medium. A: lysozyme (5.0 μg/ml) and plakin (1/3); B: lysozyme alone (5.0 μg/ml); C: plakin alone (1/3); D: no enzyme added. Morphological changes are shown in Table 2.

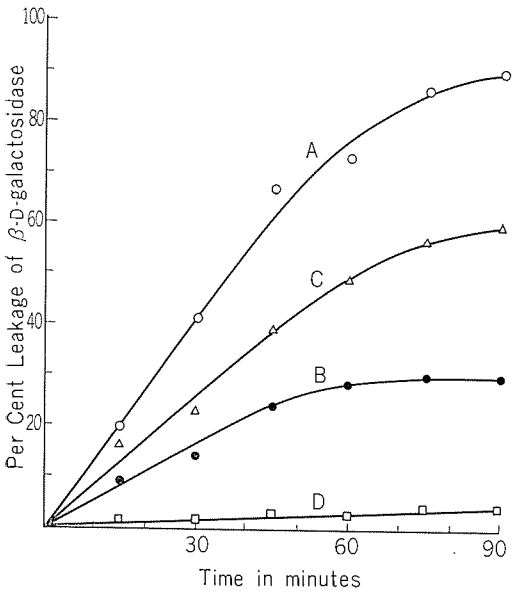

FIGURE 3 Leakage of β-D-galactosidase from BAC′ cells in hypertonic medium (12% sucrose added). Conditions are similar to those for Fig. 2.

TABLE 2 *Morphological changes during immune bacteriolysis* (Fig. 2 and 3)

Expt.	min	15	30	45	60	90
020668-1 (isotonic)	A	2+	3+	3+	4+	4+★
	B	2+	3+	3+	4+	4+
	C	—	—	—	★	★
	D	—	—	—	—	—
020668-2 (hypertonic)	A	2+	3+	3+	4+	4+★
	B	2+	3+	3+	4+	4+
	C	—	—	—	★	★
	D	—	—	—	—	—

For explanation see text.

the two enzymes were also observed.

As mentioned before, in medium made hypertonic with sucrose there was no leakage of intracellular enzymes from bacteria during immune bacteriolysis (AMANO *et al.*, 1956b). Therefore, the effect of hypertonicity was investigated in a parallel experiment.

The second portion of the bacteria used in the previous experiment was washed once more with 12% sucrose in GPB-saline. The bacteria were converted to BAC′ under the same conditions as in the previous experiment except for the addition of sucrose to a concentration of 12%. The BAC′ cells thus formed were treated similarly to those in the previous experiment in the presence of 12% sucrose. The value for 100% enzyme activity was measured after addition of 12% sucrose to lyzed bacteria to correct for the inhibitory effect of sucrose.

As shown in Fig. 3 the leakage of the enzyme was less than in the previous experiment, especially when lysozyme was used. With the enzyme concentrations employed, lysozyme was usually more effective in promoting leakage of β-D-galactosidase in isotonic medium than plakin, while the latter was more effective than the former in hypertonic medium. These facts reflect the locations of the substrates of the respective enzymes; plakin mainly attacks the cytoplasmic membrane of BAC′, and lysozyme attacks the rigid mucopeptide layer of the cell wall resulting in mechanical destruction of the fragile cytoplasmic membrane.

As mentioned before, in the immune-bacteriolytic system plakin makes bacteria non-stainable just like protplast ghosts (AMANO *et al.*, 1954a). If lysozyme is eliminated from the reaction system, plakin converts BAC′ to non-stainable rod-shaped ghosts, as shown in Fig. 7. These facts suggest that plakin does not act on the rigid structure of the cell wall but it attacks the cytoplasmic membrane of BAC′ directly.

3. *Leakage of alkaline phosphatase, a surface-bound enzyme, during immune bacteriolysis*

Recently, the existence of a group of surface-bound enzymes in some of enteric bacteria has been demonstrated. These enzymes are thought to be present in the peripheral space, i.e., between the outer surface of the cell wall and the cytoplasmic membrane of the bacteria. They include alkaline phosphatase, acid hexose phosphatase, cyclic phosphodiesterase, 5′-nucleotidase, ribonuclease I, DNA endonuclease I and UDPG and ADPG pyrophosphatases of

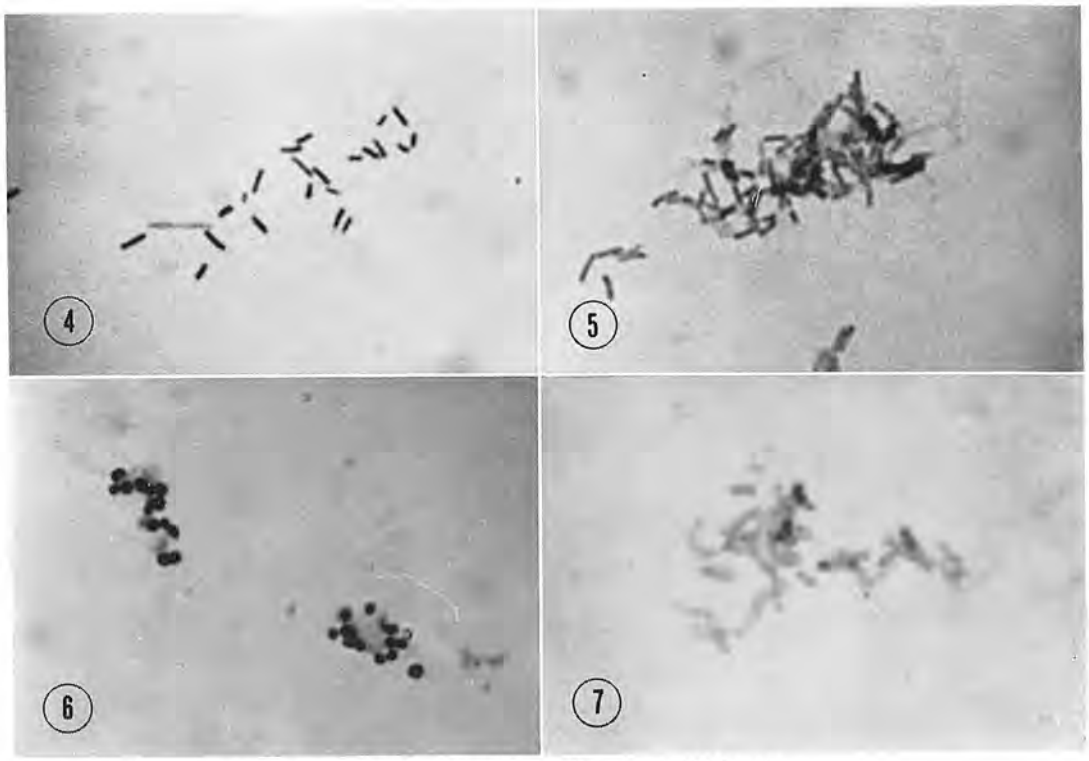

FIGURE 4 Normal cells of *E. coli* B/SM, strain 004.

FIGURE 5 BAC′ cells.
Prepared as mentioned in the experiment shown in Fig. 2.

FIGURE 6 Serum spheroplasts.
BAC′ cells were treated with lysozyme at a concentration of 5.0 μg/ml at 37°C for 60 min. The spheroplasts show 4+ morphological change.

FIGURE 7 Plakin-treated BAC′.
BAC′ cells were treated with plakin at a concentration of 1/3 at 37°C for 150 min. BAC′ show ★ ★ ★ ½ morphological changes.

E. coli (MALAMY and HORECKER, 1961, 1964; NEU and HEPPEL, 1964, 1965; MELO and GLASER, 1966). They are released from the bacteria by the action of lysozyme and EDTA during spheroplast formation. They are also released by osmotic shock on transfer of bacteria from hypertonic sucrose-EDTA medium to hypotonic solution. The exact anatomical location of these enzymes is not clear in relation to the structure seen in electron-micrographs of ultra-thin sections of the bacteria, but they are present outside the cytoplasmic membrane within which are there intracellular enzymes including β-D-galactosidase. It is, therefore, very interesting to see what happens to surface-bound enzymes during immune bacteriolysis.

E. coli B/SM, strain 004P$^+$5 was grown overnight in Tris medium at 37°C. The bacteria were harvested by centrifugation, and were washed twice with GPB-saline and resuspended in the same buffer at a concentration of 1.0×10^9 bacteria/ml.

At time, 0, the bacterial suspension was poured into flasks containing four volumes of reaction mixture at 37°C. The reaction mixture contained 1/500 lysozyme-free antiserum, 1/150 RL (3) and the bacteria at a concentration of 2.0×10^8/ml in GPB-saline (Flask A). In addition 5.0 μg of lysozyme/ml were added to Flask B and 1/5 plakin to Flask C. Flasks D, E and F were similar to Flasks A,B and C, respectively, except that they contained heat-inactivated RL (3) instead of RL (3). At intervals, a 1.5 ml aliquot was taken from each flask and was

centrifuged immediately. Enzyme activity was measured in the supernatant.

In contrast to the similar experiment on β-D-galactosidase (Fig. 1), alkaline phosphatase leakage due to antibody and RL occurred in the absence of either lysozyme or plakin, as shown in Fig. 8. Addition of lysozyme or plakin to the reaction system accelerated this leakage. The greater accelerating affect of plakin might be due either to the localization of alkaline phosphatase on or near the cytoplasmic membrane or to some spatial relation of this enzyme to phospholipids in the peripheral space of bacteria.

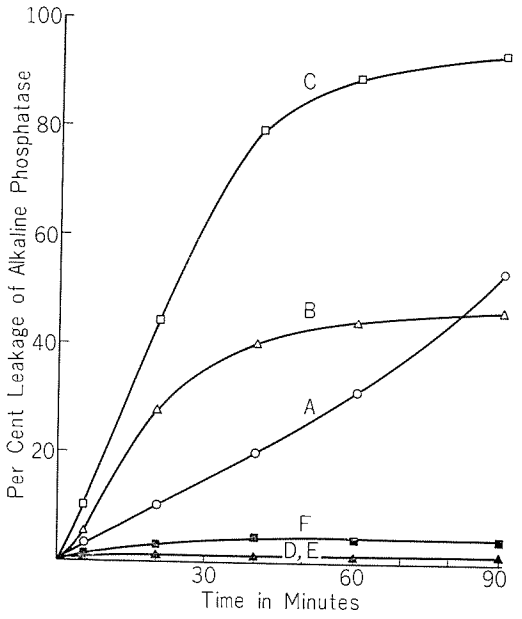

FIGURE 8 Leakage of alkaline phosphatase during immune bacteriolysis. A: lysozyme-free system; B: lysozyme (5.0 μg/ml) added; C: plakin (1/5) added; D, E and F: controls of A, B and C, respectively, with heat-inactivated RL instead of RL. Morphological changes are shown in Table 3.

TABLE 3 *Morphological changes during immune bacteriolysis* (Fig. 8)

min	15	30	45	60	90
A	—	—	—	—	—
B	2+	3+	4+	4+	4+
C	—	—	—	↑	★
D	—	—	—	—	—
E	—	—	—	—	—
F	—	—	—	—	—

DISCUSSION

Formation of serum spheroplasts by immune bacteriolysis needs lysozyme in addition to antibody and all nine components of complement (INOUE *et al.*, 1959, 1968). When serum spheroplasts are formed in appropriate hypertonic medium, they retain most of their physiological activities such as respiration, synthesis of inducible enzymes and multiplication of bacteriophage (AMANO *et al.*, 1956b, 1957). In normal isotonic medium, however, these activities are lost, probably due to mechanical destruction of the fragile cytoplasmic membrane, which, in normal bacteria, is supported by the rigid cell wall (AMANO *et al.*, 1954b, 1955, 1956a). The serum spheroplasts formed in normal isotonic medium, however, do not lose intracellular nucleic acids, which can be liberated quantitatively into the surrounding medium by treatment with 0.05% sodium deoxycholate (AMANO *et al.*, 1958).

If the bacteria are treated with lysozyme-free antibody and RL, the resultant cells, i.e., BAC′, do not change morphologically, as shown in Fig. 5. They also lose little of their intracellular enzymes, such as β-D-galactosidase. They are not susceptible to the lytic action of sodium deoxycholate, but they lose their colony-forming ability (INOUE *et al.*, 1959). Moreover, as shown in this paper, they gradually lose surface-bound enzymes, such as alkaline phosphatase, which are believed to be present inside the outer surface of the cell wall and outside the cytoplasmic membrane.

Lysozyme cannot act on its substrate, the polysaccharide backbone of the rigid mucopeptide layer, of gram-negative bacteria except under certain conditions, such as in the presence of EDTA at a certain pH. However, it can penetrate the cell wall of BAC′ and act on its

substrate resulting in the destruction of the rigid layer and formation of spheroplasts (Fig. 6). The serum spheroplasts thus formed lose their intracellular β-D-galactosidase, probably due to mechanical destruction of the fragile cytoplasmic membrane which is no longer protected by the rigid cell wall.

Plakin was discovered as an anthracidal substance in normal horse and rabbit blood platelets (GRUBER and FUTAKI 1907). It has been demonstrated that plakin acts on bacteria of the genus *Bacillus*, and destroys their protoplast membrane (AMANO *et al.*, 1956c). Recently, it has been found that plakin is a phospholipase A (HIGASHI *et al.*, 1966). Although plakin alone cannot attack usual gram-negative bacteria, it converts them to non-stainable ghosts, when it is introduced into the immune-bacteriolytic system (AMANO *et al.*, 1954a). If plakin is added to a lysozyme-free immune-bacteriolytic system, the bacteria are converted to non-stainable rods, as shown in Fig. 7. Therefore, although plakin alone cannot penetrate the cell wall of gram-negative bacteria, it passes through the cell wall of BAC′ and directly attacks the cytoplasmic membrane without affecting the rigid structure of the cell wall. It has also been demonstrated that after treatment with plakin BAC′ cells liberate intracellular nucleic acids, even before treatment with sodium deoxycholate (INOUE *et al.*, 1960).

These results suggest that antibody and complement act on the cell wall of gram-negative bacteia, which is a barrier to some macromolecules, and make " holes " or " channels " in it. Surface-bound enzymes, such as alkaline phosphatase can leak out through these " channels ". Extraneous enzymes can also pass in through these " channels " to their respective substrates. That leads to spheroplast formation with lysozyme, and formation of rod-shape ghosts with plakin, a phospholipase A. The same effect was found using phospholipase A from *Habu* venom purified by diethylaminoethyl cellulose chromatography (unpublished data).

Recently, the surface lesions caused by immune bacteriolysis were demonstrated by electron microscopy even in a lysozyme free system (BLADEN *et al.*, 1966). These lesions may correspond to " channels " in the cell wall of BAC′, through which enzyme proteins can pass.

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