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CHEMICAL STUDIES ON DAMAGES OF *ESCHERICHIA COLI* BY THE IMMUNE BACTERICIDAL REACTION.
II. RELEASE OF PHOSPHATIDYLETHANOLAMINE FROM A PHOSPHOLIPASE A-DEFICIENT MUTANT OF *E. COLI* DURING THE IMMUNE BACTERICIDAL REACTION

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SUMMARY When an antibody-sensitized, phospholipase A-deficient mutant of *Escherichia coli* B/SM was treated with complement in the absence of lysozyme, bacterial phosphatidylethanolamine (PE) was liberated into the lipid fraction of the surrounding medium, but only traces of its degradation products were found in this fraction.

Therefore, most of the degradation of bacterial PE to FFA and LPE observed in the usual immune bactericidal reaction (Inoue et al., 1974) must be the result of the action of bacterial phospholipase A which is activated or becomes accessible to its substrate on formation of lesions by complement.

The mechanism of complement-mediated formation of membrane lesions is discussed on the basis of these results.

INTRODUCTION

The preceding paper (Inoue et al., 1974) showed that bacterial phospholipids or fragments containing phospholipid were liberated into the surrounding medium when bacteria were treated with antibody and complement. In the immune bactericidal reaction, phosphatidylethanolamine (PE) appeared first in the lipid fraction of the surrounding medium, and then both free fatty acids (FFA) and lysophosphatidylethanolamine (LPE) appeared and increased gradually in this fraction.

This degradation of bacterial phospholipids seems to be due to a phospholipase A activity. There are four possible source of this enzyme. (a) It could be formed or activated directly from some complement components when they are activated. (b) It could be a serum phospholipase A other than a complement com-

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ponent which is activated or gains access to its substrate when complement is activated on the bacterial surface. (c) It could be a bacterial phospholipase A which is activated during the immune bactericidal reaction. Or (d) bacterial phospholipase A may gain access to bacterial phospholipids when the bacteria are attacked by complement.

To examine the problem phospholipase A-deficient mutants were isolated from *E. coli* B/SM (Inoue and Amano, 1974). In this work, the alteration of phospholipids in one of these mutants during the immune bactericidal reaction was compared with that in the parent strain.

MATERIALS AND METHODS

The medium, antiserum, complement and other materials and the methods used for lipid extraction and for measuring radioactivity were as described in the preceding paper (Inoue et al., 1974).

E. coli B/SM, strain 1-1 was described in the preceding paper (Inoue et al., 1974). Phospholipase A-deficient mutants were isolated from this strain following treatment of the bacteria with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described in a separate paper (Inoue and Amano, 1974). The strains were cultured and labelled with ^{14}C as described in the preceding paper (Inoue et al., 1974).

Immune bactericidal micro-test

This was performed using Microtiter plates (Cooke Engineering Co., Alexandria, Va.). Lysozyme-free antiserum was diluted serially with Tris buffered saline containing 1.0 mM $MgCl_2$ and 0.15 mM $CaCl_2$ (TBS^{++}), and 1 drop (0.025 ml) was put in each well of the plate with 1 drop of TBS^{++}, 1 drop of bacterial suspension at a concentration of 5×10^5 bacteria/ml in TBS^{++} and 1 drop of lysozyme-free complement serum (RL) diluted serially in TBS^{++}. The plate was incubated on a vibrator at 37 C for 60 min. After incubation, each reaction mixture received 1 drop of 1 mg/ml streptomycin dissolved in 5-fold concentrated tripticase soy broth (5 × conc. TSB). The plate was vibrated for a few minutes and then covered with a sheet of polyethylene film and incubated overnight at 37 C without shaking.

TABLE 1. *Immune bactericidal activity[a] of lysozyme-free antiserum and RL on E. coli B/SM ana its phospholipase A-deficient mutants[b]*

Strain	E. coli B/SM, 1-1				C3PLAL				D4PLAL				B6PLAL			
RL Dilution Added	1/30	1/60	1/120	1/240	1/30	1/60	1/120	1/240	1/30	1/60	1/120	1/240	1/30	1/60	1/120	1/240
Antiserum dilution added																
1/50	0	2	3	4	0	2	2	4	0	2	3	4	0	1	3	4
1/100	0	2	2	4	0	1	2	4	0	1	3	4	0	1	3	4
1/200	0	Tr.	2	4	0	0	2	4	0	0	3	4	0	1	3	4
1/400	0	0	2	4	0	0	2	4	0	0	3	4	0	0	2	4
1/800	0	0	2	4	0	0	2	4	0	0	2	4	0	0	2	4
1/1600	0	0	2	4	0	0	1	4	0	0	1	4	0	0	2	4
1/3200	0	0	Tr.	4	0	0	Tr.	4	0	0	Tr.	4	0	0	1	4
1/6400	0	Tr.	1	4	0	0	Tr.	4	0	0	Tr.	4	0	0	Tr.	4
1/12800	0	Tr.	2	4	0	0	1	4	0	0	1	4	0	0	2	3
1/25600	Tr.	2	3	4	Tr.	Tr.	2	3	Tr.	Tr.	2	4	2	Tr.	3	3
1/51200	2	3	3	4	2	3	3	3	1	2	2	4	2	2	3	3
0	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

[a] The titration procedure was as described in the Materials and Methods.

[b] 4 to 1: Grades of growth. Tr.: Less than 10 colonies observed. 0: No growth.

RESULTS

1. *Susceptibility of phospholipase A-deficient mutants to the immune bactericidal system against the parent strain*

The mutant strains agglutinated with rabbit antiserum to the parent strain and gave the same titer as the parent (Inoue and Amano, 1974).

The mutant strains (C3PLAL, D4PLAL and B6PLAL) were tested for their susceptibility to the immune bactericidal system against the parent strain, as described in the Materials and Methods. As shown in Table 1, all three mutants tested were killed as effectively as the parent strain by RL and antiserum against the parent strain.

2. *Release of ^{14}C from a phospholipase A-deficient mutant (D4PLAL) in the immune bactericidal reaction*

The mutant (D4PLAL) and the parent strain were each labelled with ^{14}C, sensitized with lysozyme-free antiserum, and then treated with RL. As controls, samples were treated with inactivated RL (⊿RL). After incubation at 37 C for 60 min, the reaction mixtures were centrifuged, and the supernatants and precipitates were extracted with chloroform-methanol, as described previously (Inoue et al., 1974).

Table 2 shows the distribution of ^{14}C between the supernatants and precipitates and in the fractions obtained from them.

The release of ^{14}C from the mutant strain into the supernatant was similar to that from the parent strain, but ^{14}C in the lipid fraction (chloroform phase) of the supernatant from the mutant was less than that in the lipid fraction from the parent strain.

The lipid fractions were chromatographed on an aluminum sheet of silica gel with chloroform-methanol-water (65: 25: 4, by vol.). The autoradiogram in Fig. 1 shows that the lipid fraction of the supernatant from the RL-treated parent strain (As) contained free fatty acids (FFA) and lysophosphatidylethanolamine (LPE) in addition of a minute amount of phosphatidylethanolamine (PE), as described in the preceding paper (Inoue et al, 1974). The lipid fraction of the supernatant from the mutant strain (Cs), on the other hand, contained a small, but significant amount of PE and only traces of FFA and LPE with a trace of phosphatidyl glycerol.

3. *Alteration of phospholipid in the pholipase A-deficient mutant strain when treated with a large amount of RL*

As described above, the lipid fraction released from the RL-treated mutant still contained traces of FFA and LPE. To increase the reaction, 1/2.5 RL was used with

TABLE 2. *Percent distribution of ^{14}C of E. coli B/SM and a phospholipase A-deficient mutant after treatment with lysozyme-free antibody and RL*

Stain	1-1		D4PLAL	
Treated with	Ab+RL	Ab+⊿RL	Ab+RL	Ab+⊿RL
Supernatant (Sup.)	7.7	2.1	6.22	1.35
Precipitate (Ppt.)	92.3	97.9	93.8	98.6
Sup.				
Chloroform phase	0.49	0.09	0.097	0.057
Methanol-water phase	2.16	0.98	3.7	0.83
Ppt.				
Chloroform phase	11.0	12.2	11.3	11.2
Methanol-water phase	2.6	3.46	2.36	5.34

6.0×10^9 bacteria/ml instead of 1/20 to 1/25 RL with 8.0×10^9 bacteria/ml, as in the standard reaction mixture. As shown in Fig. 2, the lipid fraction of the supernatant from the mutant (Cs) showed distinct spots of both FFA and LPE, although they were smaller than the spot of PE of the same fraction.

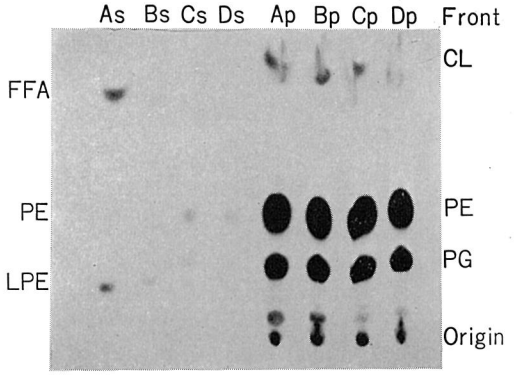

FIGURE 1. *Autoradiogram of thin layer chromatogram of the lipid fractions of the supernatants and precipitates from E. coli B/SM and a phospholipase A-deficient mutant after treatment with lysozyme-free antibody and RL.*

The lipid fractions were the same to the chloroform phase of the experiment in Table 2. Chromatography was carried out on aluminum sheet silica gel (Merck 5553) with chloroform-methanol-water (65: 25: 4, by vol.). A and B, parent strain 1–1; C and D, mutant strain D4PLAL; A and C, immune bactericidal (RL) systems; B and D, control (ΔRL) system. The subscripts s and p indicate the supernatant and precipitate, respectively. FFA, free fatty acids; PE, phosphoatidylethanol amine; LPE, lysophosphatidylethanolamine; CL, cardiolipin; PG, phosphatidylglycerol.

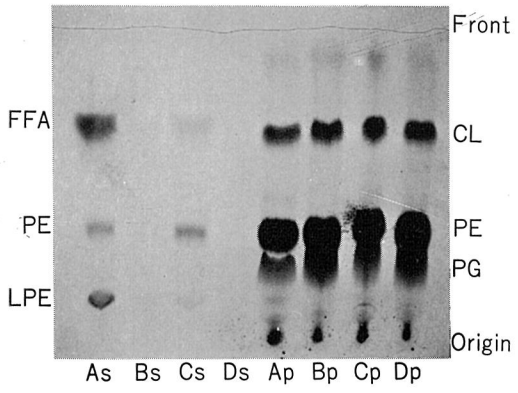

FIGURE 2. *Autoradiogram of thin layer chromatogram of lipid fractions from the parent and mutant strain treated with lysozyme-free antibody and excess RL.*

Chromatography was performed on silica gel H on a glass plate with chloroform-methoanl-water (65: 25: 4, by vol.). A and B, parent strain 1–1; C and D, mutant strain D4PLAL; A and C, immune bactericidal (RL) systems; B and D, control (ΔRL) system. The subscripts s and p indicate the supernatant and precipitate, respectively.

DISCUSSION

As shown in the preceding paper (Inoue et al., 1974) during the immune bactericidal reaction, bacterial PE and its degradation products, FFA and LPE, appear in the lipid fraction of the surrounding medium, and the degradation products increase gradually. Smith and Becker (1968) also reported an increase of titrable acid and a decrease of serine or choline phospholipid in the reaction mixture during immune hemolysis. On the other hand, Inoue and Kinsky (1970) demonstrated that no enzymatic degradation of membrane phospholipid occurred during formation of lesions in liposomes, which are artificial phospholipid-containing membranes, formed by the action of the complement.

To determine the source of the phospholipase A activity in the immune bactericidal reaction phospholipase A-deficient mutants were isolated from *E. coli* B/SM. The mutants obtained were all susceptible to the bactericidal activity of the complement and antibody against the parent strain, even in the absence of lysozyme, as shown in Table 1. It was found that a similar amount of ^{14}C was liberated from one of these mutant strains

during the immune bactericidal reaction as that liberated from the parent strain. The amount of isotope in the lipid fraction liberated from the mutant, however, was shown to be significantly less than that liberated from the parent strain. Most of the lipid found in the supernatant of the complement-treated mutant was PE, while only traces of FFA and LPE were observed. The presence of the latter two compounds in the supernatant was clearly seen when the mutant was treated with a high concentration of RL.

It is uncertain whether this degradation of phospholipids in the phospholipase A-deficient mutant strain is due to (1) the direct action of complement, or (2) to a serum phospholipase A other than complement components, which attacks its substrates at or near the lesions formed by the complement, or (3) to a minor bacterial phospholipase A which is still present after this mutant has lost most of its phospholipase A's. However, the degradation in the mutant is very small compared to that in the parent strain. Therefore, most of the degradation of bacterial PE to FFA and LPE observed in the usual immune bactericidal reaction must be the result of a bacterial phospholipase A which is activated or gains access to its substrate on formation of lesions by the complement.

When *E. coli* is infected and lyzed by bacteriophage T4, FFA is also formed (Cronan and Wulff, 1969). In this case, however, most of the FFA formed remains in the bacterial cells (Inoue and Amano, 1974). This is very different from the case in the immune bactericidal reaction, where FFA is liberated into the lipid fraction of the surrounding medium.

Complement can cause liberation of PE or material containing PE from the antibody-sensitized phospholipase A-deficient mutant even in the absence of lysozyme, as shown in this paper.

Based on the differences between the releases of enzymes in the periphery of cells and the releases of intracellular enzymes during the immune bactericidal reaction, we postulated that complement produces " channels " in the outer membrane of the cell wall of sensitized Gram-negative bacteria and that macromolecular substances, such as lysozyme and enzymes in the periphery of the cells, can pass in or out through these channels (Inoue et al., 1968).

Recently Mayer (1972, 1973) proposed a " doughnut " theory as the mechanism of cytolysis by the complement. According to this theory, late-acting components C5-C9 aggregate to form a hollow cylinder or a " doughnut " on the surface membrane of the cell when complement components are activated. The " doughnut " sinks into the cell membrane with its hydrophobic portion outwards and a hydrophilic annular space in the center. Water and ions flow into the cell through this hydrophilic hollow space and eventualy the cell bursts.

It was formerly suspected that phospholipids are only present in the cytoplasmic membrane of Gram-negative bacteria. However, it was recently demonstrated that they are also present in the outer membrane of the cell envelope. It was also shown that phospholipases, such as phospholipase A and lysophospholipase, are localized in this outer membrane (Osborn et al., 1972).

It is uncertain how the " doughnut " sinks into the cell membrane, or how the " channel " is formed in the outer membrane of Gram-negative bacteria. The " doughnut " might thrust aside membrane phospholipid because its outside is hydrophobic. The results obtained in this work suggest that the complement might quarry PE or fragments containing PE from the outer membrane of Gram-negative bacteria which become susceptible to the action of bacterial phospholipase A. This would result in formation of lesions or " channels." The bacterial phospholipase A also exerts its activity on the substrate present in a limited area near the lesion, resulting in increased liberation of FFA and LPE.

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