DEGRADATION OF PHOSPHOLIPID IN *PSEUDOMONAS AERUGINOSA* INDUCED BY POLYMYXIN B

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Effects of polymyxin B on the synthesis and degradation of lipid, ribonucleic acid (RNA) and protein in *Pseudomonas aeruginosa* were investigated. It was found that polymyxin B caused a marked degradation of the lipid fraction which was pre-labeled with (^{3}H-2)-glycerol. Thin-layer chromatographic analysis indicated that the main degraded lipids were phosphatidylethanolamine and phosphatidylglycerol, which constituted 80% and 15% of the total phospholipids of this organism, respectively. Polymyxin B also inhibited synthesis of RNA and protein *in vivo*. The severe inhibition of the uptake of labeled amino acids by polymyxin B indicated that the observed inhibition of RNA and protein synthesis possibly occurred at the level of substrate transports. The degradation of phospholipid might account for the defective membrane activities.

Polymyxins are cyclic peptide antibiotics produced by *Bacillus polymyxa*. Gram-negative bacteria are generally more sensitive to polymyxins than Gram-positive bacteria. In 1950's, NEWTON[1] showed that polymyxin interacted with a component on cell surface and caused the leakage of cell constituents, which was presumably associated with its bactericidal action. Since then, a number of reports have appeared and most of them suggested that the bactericidal action of polymyxin is due to its binding with phospholipid of the cell envelope.[2~5] However, the precise mode of action of polymyxin still remains unknown. So far only a few investigations were reported concerning with the effects of polymyxin on the metabolism of macromolecules in bacteria.[6,7]

The present report describes the effects of polymyxin B on the synthesis and degradation of lipid, RNA and protein in *Pseudomonas aeruginosa* 6073, which is very sensitive to this antibiotic. The results show that polymyxin B causes a marked breakdown of phospholipid, especially phosphatidylethanolamine (PE) and phosphatidylglycerol (PG).

Materials and Methods

Bacteria, medium and growth conditions:

Pseudomonas aeruginosa 6073 from the collection of this laboratory was used throughout this study. The organism was grown at 30°C with shaking in a nutrient broth (pH 7.0) containing 0.5% NaCl, 1% Polypeptone (Daigo Eiyo Kagaku Co., Ltd., Osaka), and 1% meat extract (Kyokuto Seiyaku Co., Ltd., Tokyo). The overnight culture was diluted with 100 volumes of the same nutrient broth medium and was incubated at 30°C on a reciprocal shaker at 120 r.p.m.. Bacterial growth was measured by optical density (O.D.) at 660 nm using a Hitachi photoelectric colorimeter (FPW-4).

Chemicals:

Carrier-free (^{32}P)-phosphate, (^{3}H-2)-glycerol (200 mCi/m mole), (^{3}H)-L-phenylalanine (5.39 Ci/m

mole) and (^{3}H)-uracil (18.4 Ci/m mole) were purchased from the Daiichi Pure Chemicals Co., Ltd., Tokyo. ^{14}C-Amino acid mixture (*Chlorella* protein acid hydrolysate) was supplied from the Institute of Applied Microbiology, University of Tokyo. Polymyxin B (PLB) was purchased from the Sigma Chemical Company, St. Louis, Mo., U.S.A. Tetracycline and chloramphenicol were the products of the Takeda Pharmaceutical Industries, Ltd., Osaka and Sankyo Co., Ltd., Tokyo, respectively. Colistin sulfate was kindly provided by the Kayaku Antibiotics Research Co., Ltd. Tokyo. Other chemicals are purchased from commercial sources.

Synthesis of lipid, RNA and protein:

The cells were grown in the nutrient broth and when the O.D. of the cultures reached to 0.3, the various radioactive precursors (^{3}H-2-glycerol 0.2 μCi/ml, ^{3}H-uracil 0.2 μCi/ml or ^{3}H-phenylalanine 6 μCi/ml at final concentrations) were added. The cultures were further incubated with or without PLB (2~5 μg/ml). The reaction was terminated by adding an equal volume of 10 % trichloroacetic acid (TCA). The precipitate was collected on a Millipore filter (HAWP; pore size 0.45 μ) and was washed with 5 % TCA solution. The precipitate on the filter was placed in a glass counting vial and 0.5 ml of 5 % sodium dodecyl sulfate (SDS) was added. The vials were incubated at 30°C overnight. Radioactivity was counted by a liquid scintillation spectrometer (Horiba Model LS-500) using the scintillation fluid of the toluene-Nonione system.[8]

Rate of phospholipid synthesis:

The rate of synthesis of phospholipid was determined by the pulse labeling with (^{3}H-2)-glycerol and (^{32}P)-phosphate as follows: The cells were grown in the nutrient broth. When the O.D. of the culture reached to 0.3, the culture was separated into two portions. PLB was added to one culture at the final concentration of 5 μg/ml. Both cultures were further incubated. At time intervals, 2 ml fractions were withdrawn from the control and the PLB-treated cultures. Each fraction was incubated with 200 μCi of (^{32}P)-phosphate and 2 μCi of (^{3}H-2)-glycerol for 5 minutes at 30°C with shaking. The reactions were terminated by 5 ml of cold methanol and were subjected to phospholipid analysis. Areas corresponding to PE, PG, cardiolipin (CL) and X, which is an unidentified one, were scraped off from the thin-layer chromatography plate and their radioactivities were counted by the liquid scintillation spectrometer using a toluene scintillation fluid.

Degradation of lipid, RNA and protein:

The cells were pre-labeled with various radioactive precursors (^{3}H-2-glycerol 0.5 μCi/ml, ^{3}H-uracil 0.1 μCi/ml or ^{3}H-phenylalanine 6 μCi/ml at final concentrations) during their growth from O.D. 0.1 to 0.3 with shaking. When the O.D. of the culture reached to 0.3, the cells were harvested and resuspended in a same volume of the prewarmed spent nutrient broth with or without PLB (2~5 μg/ml), and they were further incubated. At time intervals, 0.2 ml portions of cultures were withdrawn and 0.2 ml of 10 % TCA was added. The precipitates formed were collected on the Millipore filters and were counted as above.

Degradation of phospholipid:

The cells were grown at 30°C in two separate nutrient broth media and were labeled with (^{32}P)-phosphate (10 μCi/ml) and (^{3}H-2)-glycerol (0.4 μCi/ml) during their growth from O.D. 0.05 to 0.3. When the O.D. of each culture reached to 0.3, the cells were harvested, washed once with 0.05 M phosphate buffer (pH 7.0) and resuspended in the same volume of prewarmed spent nutrient broth with or without PLB (5 μg/ml), and both cultures were further incubated. At time intervals, 2 ml of cultures were taken and the reactions were terminated by adding 5 ml of cold methanol. The mixtures were subjected to phospholipid analysis.

Extraction of phospholipid:

Phospholipids were extracted from the cultures by the modified method of BLIGH and DYER.[9] After extracting the sample with chloroform - methanol - water (10 : 10 : 9, v/v), chloroform phase was washed twice with 0.2 volume of chloroform - methanol - water (3 : 48 : 47, v/v). The washed chloroform phase was evaporated to dryness under a reduced pressure, and the

residue was dissolved in a small volume of chloroform-methanol (2 : 1, v/v).

Separation and identification of phospholipids:

Phospholipids were separated by thin-layer chromatography using the pre-coated plate, Silica gel 60 (Merck : Code No. 5724, Darmstadt, Germany). The solvent system was chloroform-methanol-acetic acid (65 : 25 : 10, v/v). For the identification of individual phospholipid, the mobility of each spot was compared with those of PE, PG, and CL extracted from *Escherichia coli*.

Results

Effects of PLB on Growth

The cells grown overnight were inoculated into the fresh nutrient broth and were grown at 30°C with shaking. At the O.D. of 0.3, various amounts of PLB were added to the cultures and were further incubated. As shown in Fig. 1, PLB inhibited the growth almost completely at 5 μg/ml and a partial inhibition was observed at 1~3 μg/ml. A higher concentration of PLB such as 10 μg/ml caused a marked lysis of the cells. Therefore, we used subinhibitory concentrations of PLB ranging from 2 to 5 μg/ml in the following experiments.

Fig. 1. Effect of PLB on growth
Growth was expressed as the optical density at 660 nm.

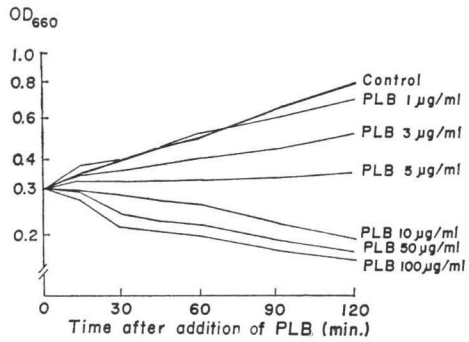
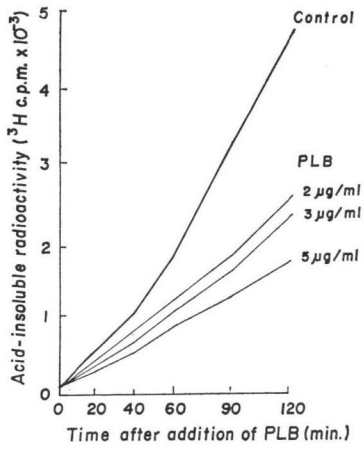


Fig. 2. Effect of PLB on lipid synthesis
Cells grown overnight were inoculated into the fresh nutrient broth containing glycerol (50 μg/ml). At O.D. of 0.3, ^{3}H-2-glycerol (0.2 μCi/ml) was added and the incubation was started.



Effects of PLB on Lipid Synthesis

Since it has been suggested that PLB binds to phospholipid, we first examined the effect of PLB on lipid synthesis.

DANIELS[10] clearly showed that (^{3}H-2)-glycerol was specifically incorporated into lipid fraction in *E. coli* and *Bacillus megaterium* since it was oxidized to dihydroxyacetone phosphate and ^{3}H-atom was lost when it entered into other metabolic systems. Therefore, it can be used as a specific lipid precursor in these bacteria. When cells of *Ps. aeruginosa* 6073 were labeled with (^{3}H-2)-glycerol, approximately 92 % of the radioactivity incorporated into the TCA-insoluble fraction was recovered in the chloroform phase after the BLIGH and DYER's extraction, which indicates that this compound can also be used as a lipid precursor in this organism. Fig. 2

shows the effect of PLB on the synthesis of lipid using (^{3}H-2)-glycerol as a tracer. Two μg/ml of polymyxin B inhibited lipid synthesis by 30 % at 60-minute and 34 % at 120-minute incubations. Therefore, the inhibition was almost comparable to that of growth.

Effect of PLB on Degradation of Lipid

As shown in Fig. 3, PLB caused a marked degradation of lipid, and approximately 50 % and 75 % of pre-labeled lipid were degraded in the presence of 3 and 5 μg/ml of PLB, respectively, after incubation for 120 minutes. When PLB-treated cultures were centrifuged at 10,000$\times g$ for 5 minutes, approximately 70~80 % of the degraded radioactivity was recovered in the supernatants and a very little radioactivity (about 4 %) of the supernatant could be extracted into the chloroform phase by BLIGH and DYER's method. This marked degradation of

Fig. 3. Effect of PLB on lipid degradation.
Time course of the loss of radioactivity from the cells (upper curves) and the increase in radioactivity in the supernatant (10,000$\times g$, 5 min) due to the leakage of cell materials (lower curves) were estimated simultaneously. Symbols for the concentrations of PLB were the same in both curves.

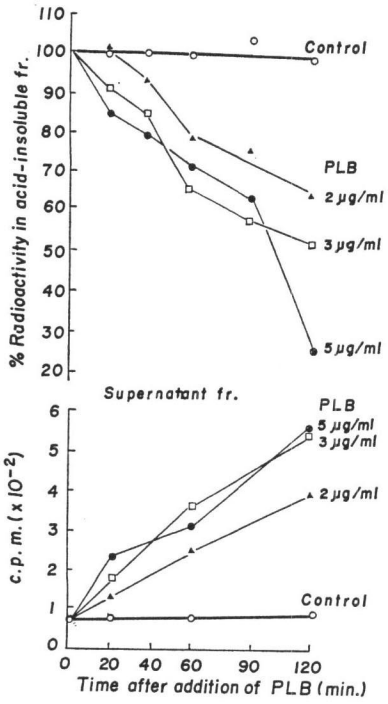
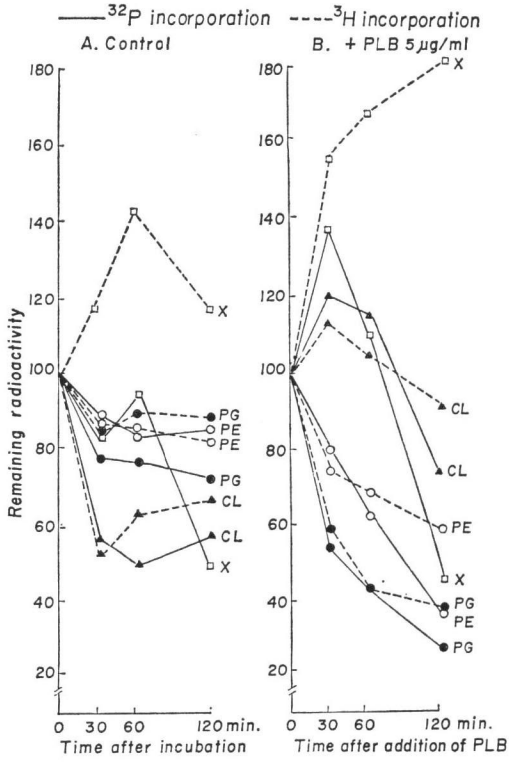


Fig. 4. Effect of PLB on phospholipid degradation of *Ps. aeruginosa* 6073.
The relative values are expressed as percentage of radioactivities at zero time.



lipid by PLB was partially prevented by adding 100 mM of $MgSO_4$. This degradation of lipid did not occur at 0°C and also in a PLB-resistant strain of *Proteus mirabilis* even at 30°C. Colistin, which is another antibiotic of the polymyxin group, had a similar lipid degrading activity at 5 μg/ml.

Effect of PLB on Phospholipid Degradation

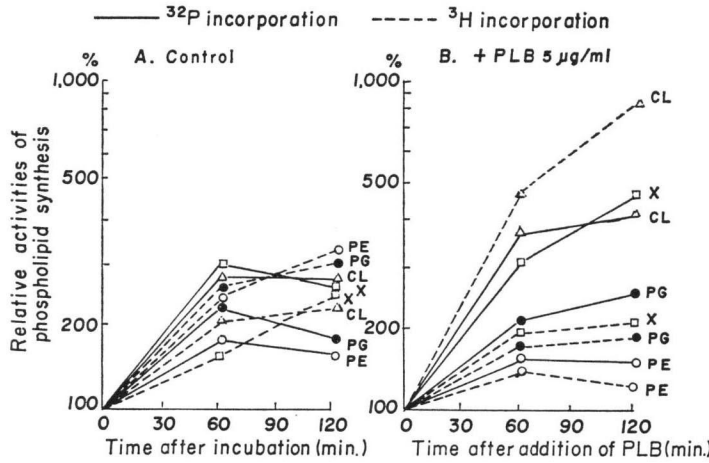
It is generally accepted that most of glycerol-containing lipids are phospholipids in Gram-

negative bacteria. Therefore, the effects of PLB on the degradation of phospholipids were examined. As shown in Fig. 4A, only 20% of PE and PG were degraded in the control culture even after 120-minute incubation, while approximately 50% of CL was degraded after the incubation. In contrast to the control, marked degradations of PE (60%) and of PG (70%) were observed in the presence of PLB after 120-minute incubation as shown in Fig. 4B. No significant degradation of CL, however, occurred in the PLB-treated culture.

Effect of PLB on the Rate of Phospholipid Synthesis

As shown above, PLB caused a marked degradation of phospholipid. Therefore, the inhibition of lipid synthesis by PLB, shown in Fig. 2, involved both the synthesis and degradation of phospholipid. In order to know the true effect of PLB on phospholipid synthesis, a short term labeling experiment, which represents the rate of synthesis, were carried out. The relative rates of four phospholipids in the control and the PLB-treated cells are shown in Figs. 5A and 5B. There are no large differences in the rates of PE and PG synthesis between the control

Fig. 5. Rates of phospholipid synthesis in the control and polymyxin-treated cultures.
The rates of phospholipid synthesis at various periods of incubation are expressed as percentage of activities at zero time.



and the PLB-treated cultures as determined by the total radioactivities. However, growth was inhibited by polymyxin and, therefore, PLB seemed to stimulate weakly the synthesis of PE and PG on the basis of their activities per cell. On the other hand, the rates of synthesis of CL and an unidentified lipid, which is termed X, were stimulated several times in the presence of PLB.

Effects of PLB on the Synthesis and Degradation of RNA and Protein

The effects of PLB on the synthesis and degradation of RNA and protein are summarized in Tables 1 and 2. As shown, protein synthesis was more susceptible to PLB than RNA synthesis as determined by the incorporation of labeled precursors. The degradation of prelabeled RNA and protein in the presence of PLB was much less than the degradation of phospholipids. Among *in vivo* synthesis of macromolecules, protein synthesis was most severely inhibited by PLB.

Table 1. Effects of PLB on RNA and protein synthesis

Fraction	Addition	Specific activity ($\times 10^{-3}$)* after addition of PLB	
		60 min	120 min
RNA	None	7.0	11.2
	+PLB (2 μg/ml)	4.6 (34)**	6.5 (42)
Protein	None	7.1	11.4
	+PLB (2 μg/ml)	4.3 (39)	6.4 (46)

* These values are calculated as follows:
c.p.m. uracil or phenylalanine incorporated into TCA insoluble fraction/Optical Density at 660 nm

** Numbers in parentheses show the percentages of inhibition.

Table 2. Effects of PLB on degradation of RNA and protein

Fraction	Addition	Radioactivity in TCA insoluble fraction (c.p.m. $\times 10^{-3}$) after addition of PLB		
		0 min	60 min	120 min
RNA	None	13.4 (100)*	13.9 (104)	13.4 (100)
	+PLB (5 μg/ml)		11.0 (82)	10.4 (78)
Protein	None	2.11 (100)	2.05 (97)	2.04 (97)
	+PLB (5 μg/ml)		2.01 (95)	1.84 (89)

* Numbers in parentheses indicate the percentages of radioactivity in TCA insoluble fraction.

In order to know the effect of PLB on protein synthesis in some detail, we investigated the effect of PLB on cell-free protein synthesis. Since the system of the cell-free protein synthesis is well established in *E. coli*, we used the extracts from *E. coli* as the enzyme source. Although tetracycline (TC) at 5 μg/ml, which is known as an inhibitor of protein synthesis, inhibited the polyphenylalanine synthesis by 50 %, neither 10 nor 100 μg/ml of PLB inhibited the synthesis but stimulated by 1.4- and 1.9-fold, respectively (data not shown). Therefore, PLB inhibits *in vivo* but not *in vitro* protein synthesis. However, in the presence of 200 μg/ml of chloramphenicol, PLB (3 μg/ml) inhibited almost completely the incorporation of ^{14}C-amino acids into the cells (data not shown). This concentration of chloramphenicol completely inhibited both growth and amino acid incorporation into the acid-insoluble fraction.

Thus, it was suggested that the inhibition of *in vivo* protein synthesis was due to the block of the transport system of amino acids by PLB.

Discussion

Although it has been suggested in numerous publications that PLB binds to phospholipid of the cell envelope,[2~5] it has been shown in this paper that PLB caused a marked degradation of phospholipids, especially PE and PG, which are the major components of phospholipids in this organism. This degradation may be considered as one of the primary action of PLB. Since most of the degradation products are water-soluble, cleavage of ester bonds between glycerol and fatty acids might occur in the presence of PLB. The preliminary study of the degradation products by gel filtration and paper chromatography suggests that one of them is probably glycerophosphorylethanolamine. Why PLB causes the degradation of phospholipid,

however, is an open question. Two possibilities are considered. One is that PLB stimulates phospholipase activity by an unknown mechanism. The other is that PLB might interact with lipoprotein of the membrane physically or chemically, in such a manner of rendering the lipid moieties are accessible to the degrading enzyme. Recently two groups[4,5] showed that PLB damages the membrane because it stimulates the release of trapped glucose or K^+ in the liposomes, a membrane system consisted of lipid spherules. These experiments might support the latter hypothesis.

The degradation of phospholipids in the cytoplasmic membrane may result in the alteration of membrane activities. In fact, the transport of amino acids was severely inhibited by PLB. The observed inhibition of protein and RNA syntheses *in vivo* may be explained by this inhibitory effect. TEUBER[7] also showed that PLB completely blocked the incorporation of ^{14}C-uracil into RNA in *Salmonella typhimurium* at 4 μg/ml.

Acknowledgement

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