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STUDIES ON IMMUNITY TO MEGACIN A: SUSCEPTIBILITY OF PHOSPHOLIPIDS TO PHOSPHOLIPASE A ACTIVITY OF MEGACIN A

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SUMMARY Comparative studies were made on phospholipids from *Bacillus megaterium* strain 216, a megacin A producer, and a more immune mutant, strain 265.

The total yields of phospholipids from the two strains were similar on the basis of bacterial dry weight.

The compositions of the components of the phospholipids of the two strains were also similar.

The phospholipase A of megacin A was found to be a phospholipase A_2.

The susceptibilities of the respective phospholipids from the two strains were also quite similar. The patterns of the unhydrolyzed fatty acids of the two lyso-phosphoglycerols were also the same.

These results do not explain the difference in the susceptibilities to megacin A of the two strains.

The importance of the megacin specific inhibitor in immunity to megacin A is discussed.

INTRODUCTION

Megacin A is an exceptional bacteriocin in the following two respects: 1) it is an enzyme (phospholipase A), hydrolyzing phospholipids to lysophospholipids (Ozaki et al., 1966) and 2) it causes lysis of sensitive bacteria (Iványovics et al., 1959). *Bacillus megaterium* strain 216 and some strains derived from it, which all produced megacin A, were relatively insensitive to megacin A as compared to strain KM (most sensitive indicator). Some strains derived from strain 216 were more sensitive than the parent, while others were less sensitive (Ochi et al., 1970). These differences in sensitivity to megacin A seem to indicate that these strains have different degrees of immunity to megacin A.

As described in the previous report (Ochi et al., 1970), the bacteria with immunity to

megacin A produce a megacin inhibitor, which reversibly inhibits megacin activity or slows down the turn-over rate of the phospholipase A activity of megacin A rather than inactivating it. The degrees of immunity were roughly proportional to the amounts of megacin inhibitor produced in the supernatants of the cultures. In addition, the immunity is retained by protoplasts and this immunity shows high specificity for the phospholipase A of megacin A but not for that of heated " Habu " venom. This highly restricted specificity in the inhibition of phospholipase A activity by the megacin inhibitor was demonstrated on an enzymic level for the activity of megacin A and not for that of heated " Habu " venom.

These facts suggest that immunity to megacin is due to production of the megacin inhibitor. However, before drawing this conclusion, another possibility must be examined, namely that some fatty acids of phospholipids in the cytoplasmic membrane of more immune strains may have a low affinity for megacin A but not for the phospholipase A of heated " Habu " venom. This paper describes results of studies on the affinities of phospholipids of strains with high and low immunity to phospholipase of megacin A.

MATERIALS AND METHODS

1. *Bacterial strains used*

Bacillus megaterium strain 216 (megacin A producer), its mutant #265 (less sensitive to megacin A, producing an inhibitor for megacin A) (Ochi et al., 1970) and strain KM (an indicator for megacin A) were used.

2. *Medium*

The P-Y medium described previously (Ochi et al., 1970) was used. It consisted of polypeptone (Daigo Nutritive Chemicals Co., Japan) 10 g, yeast extract (Daigo Nutritive Chemicals Co.) 5 g, NaCl 3 g, K_2HPO_4 0.73 g, KH_2PO_4 0.27 g and H_2O 1 liter (pH 7.2).

3. *Preparation of megacin A*

Megacin A was prepared as described by Holland (1961) with the following modifications : 1) induction was carried out using N-methyl-N'-nitro-N-nitrosoguanidine (NG). 2) megacin A was precipitated by adding acetic acid instead of N-HCl. 3) The DEAE cellulose column was eluted with a linear gradient of NaCl (0.2 M–0.5 M) in 5 mM phosphate buffer (pH 7.0) instead of by the batch elution method.

4. *Bacterial lipid preparation*

A 1% inoculum of an overnight culture was added to fresh medium and incubated at 37 C with shaking. In the middle of the logarithmic growth phase, the cells were centrifuged and washed twice with cold saline.

Bacterial lipids were extracted by the method of Folch (1957) as follows. The bacterial cells were put into more than 20 volumes of a chloroform-methanol mixture (2 : 1 v/v) and kept at room temperature overnight with stirring. Then the mixture was filtered and the filtrate was mixed with a quarter volume of saline (the proportions of chloroform, methanol and water were 8 : 4 : 3 by volume). The lower choloroform layer was separated and the upper layer was reextracted twice with about a quarter volume of chloroform. The chloroform layers were combined, concentrated and used as the bacterial lipid preparation.

5. *Thin layer chromatography (TLC) and preparation of bacterial phospholipids*

Silica gel H (Merck) was used for TLC. Bacterial phospholipids were separated using the solvent, diisobutyl ketone-acetic acid-water (40 : 25 : 5 v/v/v) (Marinetti et al. 1957). For location of phospholipids either Dittmer's reagent (1964) or methanol was used. When plates were sprayed heavily with methanol, areas containing phospholipids were seen as white spots.

After TLC, spots of phospholipid were located by washing the resulting powder with chloroform-methanol (1 : 1, v/v) on a sintered glass filter.

6. *Transmethylation of phospholipids*

After fractionation of phospholipids by TLC, the lipids were transmethylated by the method of Bowyer et al. (1963) with slight modification. The powder from TLC containing phospholipids was refluxed with 0.9 ml of conc. H_2SO_4 in 15 ml of methanol for 6 hr at 80–90 C with addition of hydroquinone, as an antioxidant, and carborundum

chips, to prevent bumping. Then 20 ml of water were added to the sample and the methyl esters of the fatty acids were obtained by two extractions with 20 ml volumes of petroleum ether. The extracts were washed twice with water and dried over a mixture of anhydrous sodium sulfate and sodium bicarbonate (4 : 1, w/w). Then, the samples were concentrated in a rotary evaporator and subjected to gas-liquid chromatography.

7. *Gas-liquid chromatography (GLC)*

The methyl esters of fatty acids were analyzed by gas-liquid chromatography using a Hitachi Perkin-Elmer gas chromatograph F6-D, equipped with a hydrogen flame ionization detector. A Golay column BDS (Hitachi) was used for gas chromatographic analyses. It is a stainless-steel column (0.50 mm × 45 m) packed with butanediol succinate. A stream of nitrogen at a pressure of 0.5 kg/cm² was passed through the column.

8. *Determination of the acyl ester content*

Samples of 0.4 ml were pipetted into 3 ml of ethanol-ether (3 : 1, v/v) and the acyl ester content was assayed by the method of Stern et al. (1953).

9. *Determination of the phosphorus content of phospholipids*

Each phospholipid sample was dried and its phosphorus content was assayed by the method of Bartlett (1959).

10. *Chemicals*

Sodium deoxycholate (DOC) was a product of Matheson Coleman & Bell Co. N-Methyl-N'-nitro-N-nitrosoguanidine (NG) was obtained from the K & K Laboratory (New York, U.S.A.). Standard samples of methylated fatty acids were products of the Applied Science Laboratories Inc. (U.S.A.).

Phospholipase A of snake (*Trimeresurus flavoviridis*) venom was prepared as described previously (Ochi et al., 1970).

RESULTS

1. *Chemical constitutions of the phospholipids of each strain*

Comparative studies of the total yields of phospholipids from the two strains were made on the basis of dry weight. The cells were harvested in the middle of the logarithmic growth phase and washed successively with saline and distilled water. Then thick cell suspensions were made and the phospholipids were extracted and estimated. Using suspensions of the two strains of equal optical density the dry weights of the cells were similar. The total yield of phospholipids from equal dry weight cells of the two strains were also almost equal, as shown in Table 1.

The bacterial lipids were separated by TLC and the different phospholipids were identified using Dittmer's reagent (1964) as spray. As

TABLE 1. *Phospholipid phosphorus in the two strains*

	Strain	
	Parent strain	Mutant #265
Phosphorus of phospholipid (μg/mg dry cells)	0.65	0.68

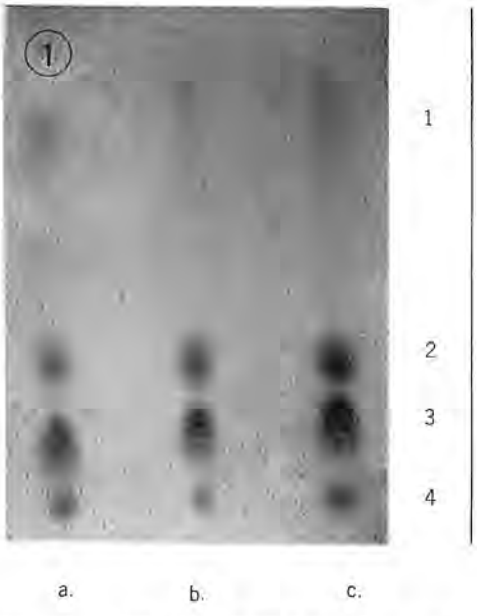

FIGURE 1. *TLC of phospholipids from B. megaterium.*
a: B. megaterium strain KM
b: B. megaterium strain 216
c: mutant 365

shown in Fig. 1, the phospholipids of the parent strain and the mutant contained four components and there was no difference between the two. Moreover, these four components were identical with those of *B. megaterium* KM, the chemical nature of which had been respectively identified by Op Den Kamp et al. (1965). So it seems likely from the TLC plate that these phospholipids were (1) cardiolipin (CL) (2) phosphatidyl-ethanolamine (PE) (3) phosphatidylglycerol (PG) and (4) lysyl-phosphatidylglycerol (lysyl-PG) in decreasing order of Rf. value.

After location with methanol, the phospholipids were scraped off and extracted successively with 3 ml, 2 ml, and 2 ml of chloroform-methanol (1 : 1, v/v). The phosphorus content of each sample was assayed as described in the " Materials and Methods ".

As shown in Table 2, the third component (PG) was the major phospholipid, and the ratios of the respective components in the two strains were the same.

TABLE 2. *Compositions of phospholipids of B. megaterium 216 and mutant #265*

Component	Phosphorus content (%)	
	Parent strain	Mutant #265
1	17	17
2	22	22
3	48	49
4	13	12
Total	100	100

2. *Fatty acid components of the phospholipids*

The bacterial phospholipids were separated by TLC and located with methanol. Then each phospholipid was transmethylated as described in the " Materials and Methods " and subjected to GLC. As shown in Fig. 2 and Table 3, iso C15 and anteiso C15 fatty acids were predominant in phosphatidylglycerol and the fatty acid constitutions of the parent strain and the mutant were the same.

Though data are not shown here, the fatty acid constitutions of the other three components were almost the same as that of PG.

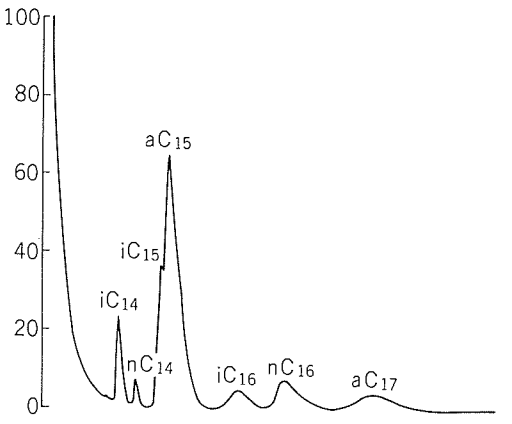

FIGURE 2. *GLC of phosphatidylglycerol from B. megaterium 216.*

TABLE 3. *Fatty acid compositions of phosphatidyl-glycerol from B. megaterium 216 and mutant #265*

Fatty acids	Area per cent	
	Parent strain	Mutant #265
i C_{14}	7	6
n C_{14}	2	2
i C_{15}	17	16
a C_{15}	53	54
i C_{16}	4	4
n C_{16}	9	10
a C_{17}	8	8
Total	100	100

Abbreviations : i, iso ; a, anteiso ; n, normal.

3. *Chemical bond hydrolyzed by megacin A*

The third component (PG) prepared by TLC was concentrated to dryness, resuspended in 0.1% DOC in 0.1 M Tris buffer (pH 7.5) and used as a substrate suspension. It was mixed with an equal volume of megacin solution (1000 units) containing 0.004 M $CaCl_2$ and incubated at 37C with shaking for 60 min. Then the lipid was extracted by the method of Folch (1957) and separated by TLC. The lyso-compound

(lyso phosphatidylglycerol) was identified by its Rf. It was extracted and subjected to transmethylation as described in " Materials and Methods " and the methyl esters were subjected to GLC. For comparison the effect of the phospholipase A of " Habu " venom was examined using 20 μg/ml of purified heated " Habu " venom in place of megacin A.

As shown in Fig. 3 and Table 4, shorter chains were removed by megacin A or by

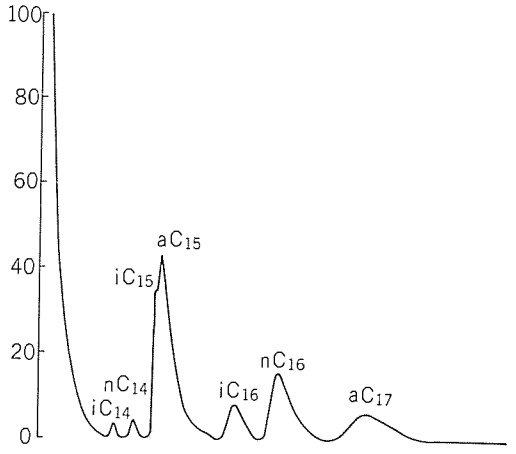

FIGURE 3. *GLC of lyso-phosphatidylglycerol prepared by megacin A.*

TABLE 4. *Fatty acid compositions of lyso-phosphatidylglycerol prepared by megacin A and " Habu " venom*

Fatty acids	Area per cent[a]	
	with megacin	with " Habu " venom
i C_{14}	0.5	0.5
n C_{14}	1	1
i C_{15}	9.5	9
a C_{15}	18	17
i C_{16}	4	4.5
n C_{16}	9	10
a C_{17}	8	8
Total	50	50

a The substrate is lyso-compound, so fatty acid compositions are calculated taking the total yield as 50%.

phospholipase A of " Habu " venom. These data indicate that megacin A hydrolyzes phospholipids at the same position as " Habu " venom. Wakui (1961) and Okuyama et al. (1965) have shown that this is ester bonds at the β-position. Therefore, the phospholipase A of megacin A is a phospholipase A_2.

4. *Hydrolysis of respective phospholipids with megacin A*

The four phospholipids were separated by TLC and concentrated to dryness. Each was then resuspended in 0.1% DOC in 0.1 M Tris buffer (pH 7.5). After centrifuging the mixtures at 4000 rev/min for 20 min, the supernatants were used as substrate suspensions.

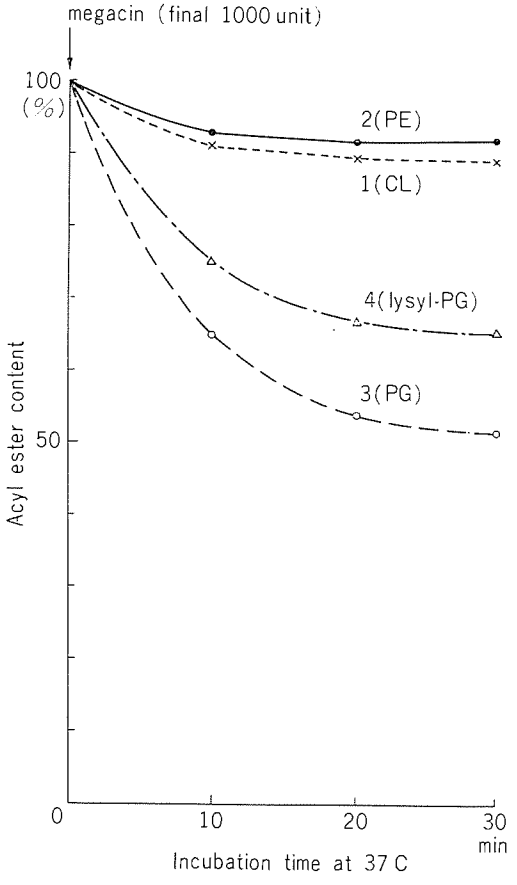

FIGURE 4. *Hydrolysis of phospholipids by megacin A.*

The acyl ester contents of the substrate suspensions were estimated and adjusted to 0.1 at OD_{525} by the method of Stern et al. (1953). The following experiments were carried out.

The substrate suspension (phosphorus content ca. 10 μg) was mixed with an equal volume of solution containing 2000 units of megacin A and 0.004 M $CaCl_2$ and incubated at 37 C with shaking. After 0, 10, 20 and 30 min. 0.4 ml samples of the reaction mixture were pipetted into 3 ml of ethanol-ether (3 : 1 v/v) and their acyl ester contents were assayed. As shown in Fig. 4, the third (PG) and fourth (lysyl-PG) components were hydrolyzed easily by megacin A. In these experiments also, we could find no difference in the affinities of the respective components of the parent strain and the mutant. Thus the phospholipids of the parent strain and the mutant seem to be the same, in spite of the difference in the sensitivities of these bacterial cells and protoplasts to megacin A (phospholipase A_2).

When about ten times more megacin was incubated with these substrate suspensions, less susceptible phospholipids (phosphatidyl-ethanolamine and diphosphatidyl-glycerol) were hydrolyzed completely giving lysocompounds. This means that no phospholipid was not susceptible to phospholipase A_2 of megacin and susceptible only to that of " Habu " venom.

DISCUSSION

Wakui (1961) demonstrated that the phospholipase A of heated " Habu " venom mainly liberated unsaturated fatty acids from egg yolk lecithin. In egg yolk lecithin these acids are mainly located at the β-position (Tattrie, 1959). Okuyama (1965) obtained similar results using cardiolipin from beef and human heart. Therefore, phospholipase A of " Habu " venom should be named phospholipase A_2 (Van Deenen, 1966; Lennarz, 1970). Using this enzyme, Higashi et al. (1966) showed that the fatty acids at the β-position of phosphatidyl-ethanolamine of *Bacillus megaterium* strain KM are mainly shorter than C_{16} acids while those at the α'-position are mainly longer than C_{14} acids. As described in this paper, phospholipase A_2 of " Habu " venom also liberated fatty acids which were shorter than C_{16} acids from phosphatidyl-glycerol and lysylphosphatidyl-glycerol of *Bacillus megaterium* strain 216 and its derivative. Phospholipase A of megacin A acts in the same way as phospholipase A_2 of " Habu " venom. This means, therefore, that phospholipase A of megacin A is also a phospholipase A_2.

The difference in the sensitivities to megacin A of the parent strain 216 and the less sensitive mutant 265 were studied as described in this paper. There was no difference in the compositions of the phospholipids of these two strains as determined by TLC after extraction by the method of Folch (1957). Furthermore, almost no difference was found in the total yields of phospholipids on the basis of optical density or dry weight of bacterial cells. These data exclude the possibilities, that the less sensitive mutant may contain another phospholipid which is not attacked appreciably by phospholipase A_2 of megacin A, or that it contains less phospholipid in the cytoplasmic membrane so that the phospholipid is less susceptible to enzymatic attack even though there was no difference in the component phospholipids.

A third possibility is that there is a difference in susceptibilities of the respective components in the parent and mutant strain to phospholipase A_2 of megacin A. However, these respective components of each class showed the same susceptibilities. Moreover, the nonhydrolyzed fatty acids in lysophosphatidylglycerol from the two strains, prepared from PG (the major component) by treatment with megacin, gave the same patterns on gas-liquid chromatography. Thus the result exclude the third possibility.

From the above date it does not seem possible to explain the difference in the sensitivities to megacin A of the parent strain 216 and the less sensitive mutant 265 on an enzyme-

substratel evel. The difference seems to be related to the abilities of the strains to produce the megacin inhibitor described by Ochi et al. (1970).

Ozaki et al. (1967) found that protoplasts from the parent strain 216, which were more insensitive to megacin A than those of non-megacinogenic mutants, still retained immunity when tested by protoplast-lysis (Ivánovics et al. 1959). Moreover this immunity was highly specific for phospholipase A_2 of megacin A, not for that of heated " Habu " venom. They suggested that some fatty acids in the β-position of phospholipids in the cytoplasmic membrane might have a low affinity for megacin A but not for the phospholipase A_2 of " Habu " venom. The phospholipids which are relatively in susceptible to megacin A are phosphatidyl-ethanolamine and diphosphatidyl-glycerol, as shown in this paper. However they were completely hydrolyzed when a large amount of megacin A were added. Thus this hypothesis is not tenable, and the immunity seems to be related to the production of a specific megacin inhibitor (Ochi et al. 1970), which inhibits megacin A but not phospholipase A_2 of " Habu " venom. This requires further study, especially in relation to the structure of the cytoplasmic membrane.

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