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Abstract

For the first time we found that cardiolipin was contained abundantly in Eschrichia coli, and we succeeded in isolating and purifying it as reported previously. With this E. coli cardiolipin a study was made on its reactivity to Wassermann antibody reagin by OGATA'S box titration, and the following results were obtained. L The purity of cardiolipin prepared from E. coli has been found to be satisfactory on the thin-layer chromatogram, by its chemical analyses and by its infrared spectrum study. 2. The composition of fatty acids of E. coli cardiolipin differed considerably from that of beef heart cardiolipin in the point that unsaturated fatty acids occupied only less than 66% in the former. Therefore, in the preparation of antigen, EtOH containing 20% tetrahydrofuran was used, which gave a clear solution, as E. coli cardiolipin did not dissolve completely in EtOH solution. 3. In the reaction made to take place with the serum from rabbit immunized with beef heart cardiolipin, E. coli cardiolipin gave almost the same reactivity to that of beef heart cardiolipin. 4. The reactivity of E. coli cardiolipin to the sera of syphilitic patients was also paretically the same as that of OGATA'S antigen, while it did not show any reactivity against the sera of normal person.

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IMMUNOLOGICAL STUDY ON CARDIOLIPIN FROM ESCHERICHIA COLI

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Since WASSERMANN found that the complement fixation test between the serum of syphilitic patient and the liver extract from congenital syphilitic foetus as hapten was positive (1), it has been demonstrated that alcohol extracts of normal human liver and various animal organs also have similar haptenic effects. PANGBORN found the alcohol extract of beef heart to be an excellent hapten in such tests, and also determined haptenic agent from the beef heart to be cardiolipin (2, 3).

As reported previously (4), after detecting a considerable amount of cardiolipin in *Escherichia coli* (*E. coli*), we succeeded in isolating and purifying *E. coli* cardiolipin. With a special interest in the serological reactivity of this *E. coli* cardiolipin, which has fatty acid composition distinct from beef heart cardiolipin, we performed some complement fixation tests with sera of rabbits immunized with beef heart cardiolipin and sera of syphilitic patients, using *E. coli* cardiolipin as a hapten. This report describes some interesting findings obtained in this study.

MATERIALS AND METHODS

Preparation of phospholipid from E. coli B: Cells of stock strain of *E. coli* B were grown at 37°C with gentle shaking on a semi-synthetic medium, for 6 hrs, and then harvested by centrifugation (4). The extraction of phospholipid was done by three successive extractions with chloroform-methanol (2 : 1). The phospholipid so obtained was purified by FOLCH's partition dialysis procedure (5).

Column chromatographic isolation of cardiolipin: $NaHCO_3$-treated silicic acid and silicic acid column chromatographies were used (6). A crude fraction containing cardiolipin was obtained by the former column and pure cardiolipin from the crude fraction by the latter, successively.

Silicic acid thin-layer chromatography: Chromatography was carried out on glass plate with a layer of silicic acid (Silica Gel G, Merck) and developed with chloroform-methanol-water (70 : 25 : 4, v/v). The plate was treated with molybdenum reagent, followed by charring.

Chemical analysis and infrared spectrum: Phosphorus was determined by ALLEN's method (7), fatty acid esters by RAPPORT and ALONZO's method (8) and vicinal OH group by photometoric measurement of periodate consumption (9). Infrared spectrum was measured in KBr pellet with a Nihon-kogaku infrared spectrometer.

Gas-liquid chromatography of fatty acid: Methyl esters of the fatty acids were prepared by heating cardiolipin with anhydrous methanol containing 0.5N HCl in a sealed ampule (10). The esters were extracted with three successive 5 ml portions of n-hexan, and the solvent was removed *in vacuo*. Gas-liquid chromatographic analysis of methyl esters was carried out on a 2 m steel column with a stationary phase of 10% polydiethylene glycol succinate on Diasolid L at 190°C.

Immunization procedure of rabbits with cardiolipin from beef heart: White rabbits were immunized by intravenous injections of freshly prepared complex of cardiolipin, lecithin, cholesterol and methylated bovine serum albumin every other day for three weeks by the method of INOUE (11). The serum was collected one week after the last injection.

Complement fixation reaction: The tests were performed by OGATA's box titration (12) with a slight modification in preparing the antigen. Cardiolipin from *E. coli*, lecithin from egg york and commercial cholesterol were mixed in the proportion of OGATA's recipe, and dissolved into ethyl alcohol with 10% tetrahydrofuran instead of ethyl alcohol since cardiolipin from *E. coli* had low solubility in alcohol. Separately, commercial OGATA's antigen from Sumitomo Chemicals Co. was used for the control.

RESULTS

Isolation and purification of cardiolipin: As already reported, a brown waxy substance in the amount of 75 mg was obtained, starting with 1.37 g of phospholipid from *Escherichia coli B* (*E. coli B*), by repeated column chromatographies of $NaHCO_3$-treated silicic acid and normal silicic acid (6). This brown waxy substance showed a single spot corresponding to the original phospholipid of cardiolipin on the thin-layer chromatogram (6). Fig. 1 shows the behaviors of this isolated cardiolipin as compared with those of

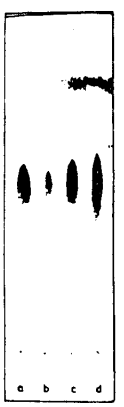

Fig. 1 Comparison of various cardiolipins on a thin-layer chromatogram. Development was in chloroform-methanol-water (70 : 25 : 4, v/v). Spots were revealed by charring after spraying with 10% H_2SO_4.

a) cardiolipin obtained from *E. coli*
b) cardiolipin obtained from beef heart
c) cardiolipin obtained from *Mycobacterium phlei*
d) synthetic cardiolipin

cardiolipin from beef heart and Mycobacterium phlei and of synthetic cardiolipin on the thin-layer chromatogram.

Chemical properties and infrared spectrum of isolated cardiolipin : The analytical data of cardiolipin from *E. coli* B showed the content of phosphorus to be 4.02% where acyl ester residue : P (molar ratio) to be 1.95 and vicinal OH group : P (molar ratio) to be 0.99. Fig. 2 illustrates infrared spectrum of the cardiolipin of *E. coli* B. As the solubility in organic solvent poses a problem in the preparation of the antigen solution for serological reaction, the solubility of the *E. coli* cardiolipin was tested with several solvents while comparing with that of beef heart cardiolipin (Table 1).

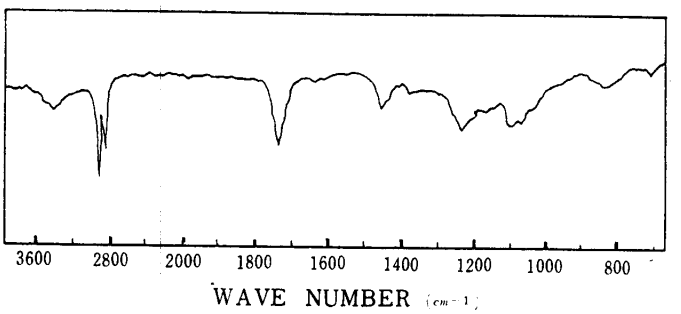

Fig. 2 Infrared spectrum (KBr disk) of cardiolipin obtained from *E. coli* B

Table 1 Solubility of Cardiolipins in Organic Solvents

Solvent / Cardiolipin of	$CHCl_3$	MeOH	EtOH	THF	EtOH with THF
Beef heart	clear	clear	clear	clear	clear
E. coli B	clear	clear	turbid	clear	clear

Note : Solubility was determined at room temperature with 1 mg of cardiolipin suspended in 1 ml of organic solvent.
Abbreviation : THF, Tetrahydrofuran

The composition of fatty acids of cardiolipin : Fig. 3 shows the gas-liquid chromatograms of fatty acid methyl esters of cardiolipin of *E. coli* B and beef heart cardiolipin. Table 2 gives the percentage of each component computed from the area of the recorded peak estimated by the triangle method. As seen in the table unsaturated fatty acids occupy 94% of the total in beef heart cardiolipin, whereas the same in *E. coli* cardiolipin less than 66%.

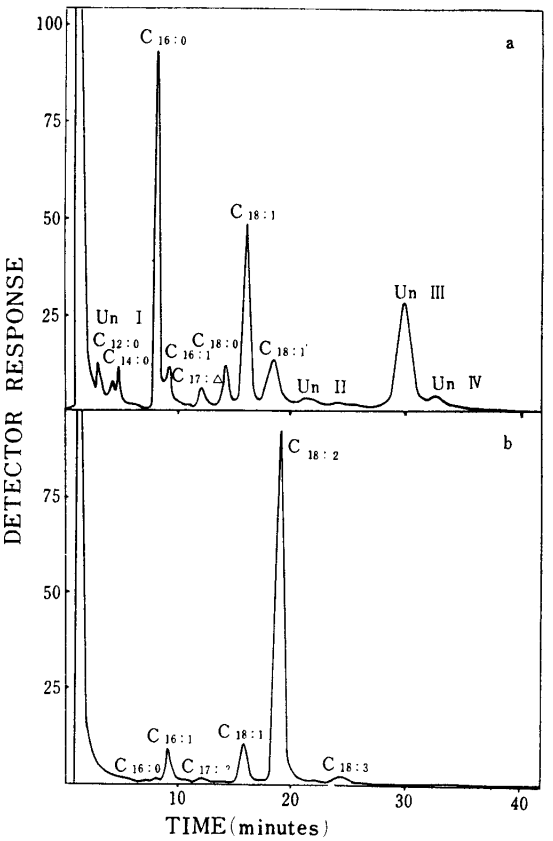

Fig. 3 Gas-liquid chromatograms of the fatty acid methyl esters of cardiolipins from E. coli B (a) and beef heart (b). Analyses of methyl esters were carried out on a 2 m steel column with a stationary phase of 10% polydiethylene glycol succinate on Diasolid L (Nikka Seiko, Co.) at 190°C.

Abbreviation: Un, Unknown fatty acid; $C_{18:1}'$, cis-Vaccenic acid

Table 2 Fatty Acid Composition of Cardiolipins from *E. coli* and Beef Heart

E. coil cardiolipin		Beef heart cardiolipin	
Fatty acid	%	Fatty acid	%
Lauric	2.1	Palmitic	5.4
Unknown I	3.7	Palmitoleic	18.4
Myristic	2.6	C_{17}: ?	5.4
Palmitic	24.1	Stearic	trace
Palmitoleic	4.0	Oleic	36.4
C_{17}-cyclic	2.8	Linoleic	25.7
Stearic	4.7	Linolenic	8.3
Oleic	20.9		
cis-Vaccenic	8.5		
Unknown II	1.7		
Unknown III	21.8		
Unknown IV	2.8		
Saturated	33.5	Saturated	5.4
Unsaturated	33.4	Unsaturated	94.2

The reactivity of E. coli cardiolipin to rabbit serum immunized with beef heart cardiolipin The reactivity of cardiolipin from *E. coli* in complement fixation reaction was tested with immunized-rabbit serum with beef heart cardiolipin by OGATA's box titration and it was compared with that of cardiolipin from beef heart. As a result the *E. coli* cardiolipin revealed the reactivity almost similar to that of the beef heart cardiolipin.

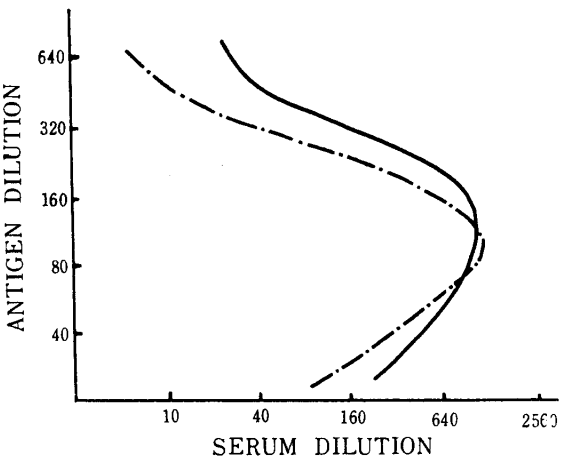

Fig. 4 Reactivity of *E. coli* cardiolipin with immunized-rabbit serum with beef heart cardiolipin in the complement fixation reaction (OGATA's box titration) The reaction was positive in the zone to the left of a curve.
——: with antigen prepared from *E. coli* cardiolipin
— · —: with antigen prepared from beef heart cardiolipin

The reactivity of E. coli cardiolipin to syphilitic sera in Ogata's box titration The syphilitic sera used were obtained from several patients in the Mizushima Kyōdo Hospital of Kurashiki City. In order to eliminate individual difference, sera from three syphilitic patients were pooled and OGATA's box titration was conducted, the results of which are shown in Fig. 4. All the antigens tested, namely, antigen from *E. coli* cardiolipin and beef heart cardiolipin, isolated and prepared by us, and commercial OGATA's antigen, showed a similar reactivity tendency. By the quantitative estimation method of the Welfare Ministry's standard (12) they all yielded antibody titers of 320x. Table 3 illustrates the results of the tests to sera from several other patients and normal persons represented in antibody titers. The features of the positive zone of the box titration were about the same in both antigens from *E. coli* cardiolipin and beef heart cardiolipin, and the results given in Table 3 were, of course, about the same. However,

both did not show any complement fixation with the sera from normal person.

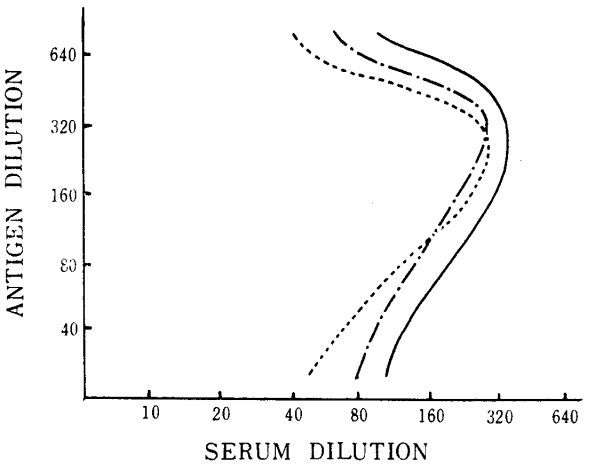

Fig. 5 Reactivity of *E. coli* cardiolipin with pooled syphilitic serum in the complement fixation reaction (OGATA's box titration)
The reaction was positive in the zone to the left of a curve.
———: with antigen prepared from *E. coli* cardiolipin
—·—·—: with antigen prepared from beef heart cardiolipin
·····: with commercial OGATA's antigen

Table 3 Comparative Titration of the Same Individuals with Different Antigens by Complement Fixation Reaction (OGATA's box titration)

	Antibody titer in serum	
	with E. coli cardiolipin	with beef heart cardiolipin
Patient A	160x	160x
Patient B	80x	120x
Patient C	320x	320x
Normal I	<2x	2x
Normal II	<2x	<2x

DISCUSSION

What WASSERMANN at first used as an antigen in the complement fixation test of syphilis was the liver extract of *Lues congenita*. At that time the component of *Treponema pallidum* in the liver was thought to have reacted with the anti-T*treponema* antibody in syphilitic sera. However, later it was found that the alcohol extracts of various organs of normal

persons or animals have such antigenic properties, and the early concept was thus overturned. Nonetheless, because this antigen is contained in a variety of organs and its nature remains unknown, it has a disadvantage that its titer can never be uniform in its preparation. Since PANGBORN later demonstrated that beef heart showed especially strong antigenicity of which cardiolipin was the essential factor (2, 3), it has become possible to use it in the quantitative reaction. After all it can be said that the true nature of Wassermann reaction is a nonspecific complement fixation reaction between cardiolipin and Wassermann's antibody reagin. The antigen used at present in the Wassermann reaction is EtOH solution composed of cardiolipin from beef heart, purified lecithin and purified cholesterol (the final concentration of each adjusted to 0.01%, 0.04% and 0.2%, respectively), which is very sensitive and readily reproducible. Cardiolipins from various animal origin have also been employed but they all proved to be inferior to that of beef heart origin quantitatively. Aside from cardiolipins of anigins, cardiolipin from soy bean was used by MURANAKA (13) in his agglutination test of syphilis.

As reported previously (4), having found as high a quantity of cadiorlipin in *E. coli* as several to 12% of total lipid, we studied the reactivity of this *E. coli* cardiolipin on the Wassermann reaction. There is a great advantage in the use of such a cardiolipin of bacterial origin, because it is possible to maintain a strict uniform condition in the culture of bacteria, hence it is possible always to supply cardiolipin of a uniform property.

The isolation and purification conducted by the rechromatography yielded highly satisfactory result. The qualitative analysis on the purity of the isolated cardiolipin has amply confirmed it to be quite pure, and also chemical assay have given the results as expected.

Infrared spectrum of *E. coli* cardiolipin coincided well with that of beef heart cardiolipin as reported by MACFARLANE (14), but the pattern of fatty acid as detected on the gas-liquid chromatograms differed considerably from that of beef heart cardiolipin. Namely, in the beef heart cardiolipin unsaturated fatty acids occupied 94% whereas those in *E. coli* cardiolipin only less than 66%, proving the latter to be not so soluble in the EtOH solution. Consequently, in the preparation of antigen, EtOH solution containing 20% tetrahydrofuran was used. In addition, the influence of such a solvent could be dismissed as the antigen prepared from beef heart cardiolipin with this solvent gave results identical with the commercial OGATA's antigen. In OGATA's box titration when the antigen prepared from cardiolipin of *E. coli* was used on syphilitic sera, the results obtained was similar to those obtained with beef heart cardiolipin. The

fact that the positive zone in Figs. 4 and 5 hardly showed any deviation even in the vertical direction implied that there was no problem in the property of *E. coli* cardiolipin as antigen as well as in its dilution stage. In the other syphilitic cases tested, identical results were obtained. In the case with sera of normal persons no complement fixation at all was detected.

A question to be asked is what factor or factors play an important role in the determination of specificity of cardiolipin in its reaction with Wassermann antibody reagin. It is said that the distance between the two phosphate groups (15, 16) and hydroxyl group on the central glycerol (17) is associated with the specificity. On this point *E. coli* cardiolipin seems to present no problem, as it has been confirmed chemically that this cardiolipin has diphosphatidyl glycerol structure, and the cross-reaction between it and the serum of the rabbit immunized with beef heart cardiolipin is clear-cut. It is said that kinds of fatty acids have no primary significance but the glyceride structure plays an important role, and our results with *E. coli* cardiolipin are in agreement with these findings.

SUMMARY

For the first time we found that cardiolipin was contained abundantly in *Eschrichia coli*, and we succeeded in isolating and purifying it as reported previously. With this *E. coli* cardiolipin a study was made on its reactivity to Wassermann antibody reagin by OGATA's box titration, and the following results were obtained.

1. The purity of cardiolipin prepared from *E. coli* has been found to be satisfactory on the thin-layer chromatogram, by its chemical analyses and by its infrared spectrum study.

2. The composition of fatty acids of *E. coli* cardiolipin differed considerably from that of beef heart cardiolipin in the point that unsaturated fatty acids occupied only less than 66% in the former. Therefore, in the preparation of antigen, EtOH containing 20% tetrahydrofuran was used, which gave a clear solution, as *E. coli* cardiolipin did not dissolve completely in EtOH solution.

3. In the reaction made to take place with the serum from rabbit immunized with beef heart cardiolipin, *E. coli* cardiolipin gave almost the same reactivity to that of beef heart cardiolipin.

4. The reactivity of *E. coli* cardiolipin to the sera of syphilitic patients was also parctically the same as that of OGATA's antigen, while it did not show any reactivity against the sera of normal person.

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