IMMUNOADJUVANT ACTIVITY OF NEGATIVELY CHARGED PHOSPHOLIPIDS ON DELAYED HYPERSENSITIVITY

Masamiki MASUI, Ikuya YANO, Gotaro YANAGIDA, Yoshinosuke TANAKA*, Shiro KUWAJIMA* and Sukeo YAMAMOTO*

Departments of Bacteriology and Internal Medicine, Osaka City University Medical School, Abeno-ku, Osaka, 545 Japan*

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1. Introduction

Immunoadjuvants hitherto known to induce delayed hypersensitivity and stimulate humoral antibody production can be classified roughly into hydrophilic and hydrophobic substances. *N*-acetylmuramyl dipeptide [1–4] and arabinogalactan peptidoglycan of mycobacteria [5–7] are examples of hydrophilic adjuvants, while Wax D [8,9], cord factor (trehalose dimycolate) of mycobacteria [10] and lipopolysaccharides of gram-negative bacteria [11] are hydrophobic adjuvants. Dailey and Hunter [12] showed the increased hydrophobicity of hapten or protein can elicit delayed hypersensitivity. On the other hand, Allison and Gregoriades [13] reported negatively charged phospholipids can elevate humoral antibody level. These investigations predict that negatively charged phospholipids might elicit delayed hypersensitivity. Our examination of literature did not reveal such lines of experiments. One of the characteristics of membrane phospholipids in halophilic bacteria is a high content of negatively charged phospholipids [14–16].

This communication reports that negatively charged phospholipids of diacyl form from *Pseudomonas halosaccharolytica* and animal tissues can elicit delayed hypersensitivity to protein antigen in guinea pigs, whereas phospholipids of the diphytanylether form from *Halobacterium halobium* are inactive. This was confirmed with synthetic phospholipids.

Correspondence should be directed to: Masamiki Masui, Department of Bacteriology, Osaka City University Medical School, Abeno-ku, Osaka, Japan 545

2. Materials and methods

An extremely halophilic bacterium, *Halobacterium halobium*, was grown in medium containing 20% NaCl at 30°C for 72 h [17] and a moderately halophilic bacterium, *Pseudomonas halosaccharolytica* ATCC 29423, was inoculated into a medium containing 10% NaCl at 30°C for 42 h in a reciprocal shaker [15]. The cells were harvested by centrifugation, washed and kept in a frozen state. Lipids were extracted three times with chloroform–methanol (2 : 1, v/v) according to the method of Folch et al. [18]. Extractable lipids were separated on DEAE–cellulose column (acetate form) chromatography by the modification of Rauser et al. [19]. Thus separated neutral and negatively charged phospholipid fractions were further separated into individual phospholipid by thin layer chromatography on Silica Gel (Merck) [16]. Quantitative estimation of each phospholipid was carried out by colorimetric determination of phosphorus. Fatty acids were analyzed by gas–liquid chromatography as methylesters as described previously [16].

Diphosphatidylglycerol and phosphatidylserine from bovine brain, phosphatidylinositol from porcine liver, synthetic dioleoyl phosphatidylglycerol and dipalmitoyl phosphatidylcholine were purchased from Serdary Research Lab. (Canada). Fully saturated phospholipids were prepared by the direct hydrogenation of bovine brain diphosphatidylglycerol in the presence of platinum oxide.

Crystalline ovalbumin (Grade V, Sigma) in saline was added to the dried phospholipids. After emulsifying with an equal volume of incomplete Freund's

adjuvant (IFA) (Difco) by sonicating for 5 min at room temperature, 0.2 ml of the water-in-oil emulsion was injected into a foot-pad of random-bred Hartley guinea pigs. Dose of antigen and phospholipids was 1 mg and 0.1 mg/animal. Wax D of *Mycobacterium tuberculosis* Aoyama B (gift of Dr A. Tanaka, Kyushu University, Japan) served as positive control, and IFA-antigen alone was used as negative control. Five animals weighing 350–400 g were used for each experiment. To test induction of delayed hypersensitivity to ovalbumin, 3 weeks after immunization, antigen solution (20 mg/ml saline) was injected into cornea of animals to make a transient disc of opacity of approx. 5 mm in a diameter (ca. 2 μl), and the resultant turbidity was evaluated 48 h later by the method of Kotani et al. [20]: 3, thickened and opaque cornea, pupil invisible; 2, diffusely turbid, pupil visible; 1, locally turbid; 0, no visible difference from uninjected cornea. Skin test was performed intradermally with 0.1 ml antigen solution (500 μg/ml saline) 3 weeks after immunization and the reaction was evaluated 48 h later. Production of serum antibodies was determined by a passive haemagglutination test 4 weeks later.

3. Results and discussion

Crude extractable lipids from *P. halosaccharolytica* induced a marked delayed hypersensitivity and elevated slightly antibody levels to ovalbumin in guinea pigs, whereas no elicitation of delayed hypersensitivity and no enhancement of antibody production were observed with the extractable lipids of *H. halobium* as shown in fig.1. The strong positive reaction in cornea continued from 24 h to 72 h and the skin test was also positive. Major components of extractable lipids in *H. halobium* are phosphatidylglycerophosphate [di-*O*-phytanyl-D-α-glycerylphosphoryl-α-glycerophosphate] (diether form), phosphatidylglycerol [di-*O*-phytanyl-D-α-glycerylphosphoryl-α-glycerol] (diether form) and glycolipid sulfate [2,3-di-*O*-dihydrophytanyl-L-glycerol-1-*O*-(glucosylmannosylgalactosyl) sulfate] [14]. In contrast, major phospholipid components of *P. halosaccharolytica* are diacyl form phospholipids, phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol and glucosylphosphatidylglycerol [15]. This strongly suggests that negatively charged phospholipids in diacyl form are useful for the elicitation of delayed hypersensitivity, whereas diphytanylether form phospholipids are inactive.

The negatively charged phospholipid fraction mainly composed of phosphatidylglycerol, diphosphatidylglycerol and glucosylphosphatidylglycerol (roughly 1 : 2.5 : 1.4, w/w) in *H. halosaccharolytica* elicited delayed hypersensitivity and elevated slightly

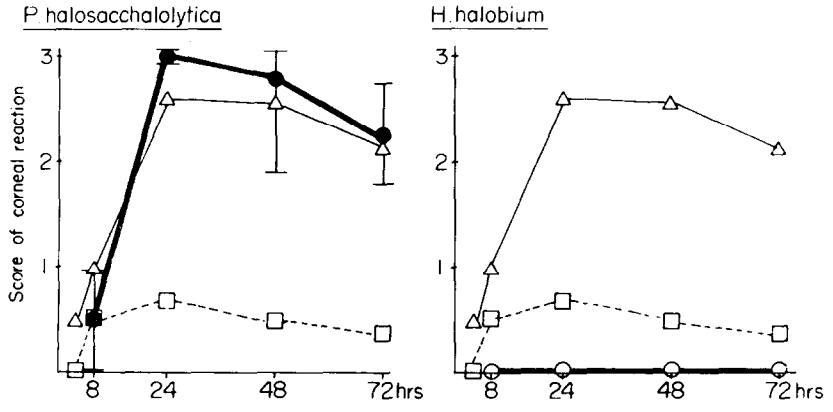

Fig.1. Inducing ability of crude phospholipid fractions of *Pseudomonas halosaccharolytica* and *Halobacterium halobium* on delayed hypersensitivity in guinea pigs. Dose of crude phospholipid fractions used for the immunization was 0.2 mg/animal. Other immunological methods, see the text. Score of corneal reaction: crude phospholipids of *P. halosaccharolytica* (●), those of *H. halobium* (○), Wax D (△), negative control (□). Mean and range of antibody titer (× log 2) in cases of crude phospholipids of *P. halosaccharolytica*, those of *H. halobium*, Wax D, and IFA alone were 14.3 (14–15), 11.6 (10–14), 16.2 (14–19), and 13 (10–14), respectively.

Table 1
Immunoadjuvant activities of phospholipids prepared from *P. halosaccharolytica* and animal tissues, and of synthetic phospholipids

Phospholipids	Ratio	Dose (mg)	Corneal reaction (mm)		Skin reaction (mm)	Antibody level (× log2)	
			Mean	Range	Mean ± s.d.	Mean	Range
Negatively charged		0.2	3.0[g]	(3.0–3.0)	10.0 ± 2.8	14.6	(13–16)
phospholipid fraction		0.1	2.4[g]	(1.0–3.0)	13.0 ± 2.9[g]	15.8	(14–17)
of *P. halosaccharolytica*		0.05	2.3[g]	(1.5–3.0)	12.0 ± 5.0[g]	14.8	(13–17)
PG[a]/PGL[a]/CL[a]/PE[a]	1 : 1.4 : 2.5 : 3	0.4	2.3	(1.0–3.0)	10.5 ± 1.9	16.3[g]	(15–17)
	1 : 1.4 : 2.5 : 3	0.2	1.9	(1.5–2.5)	8.3 ± 3.0	15.8	(13–17)
PG[a]/PGL[a]/CL[a]	1 : 1.4 : 2.5	0.2	2.1[g]	(1.5–3.0)	6.3 ± 3.8	16.0[g]	(15–17)
PG[a]/PGL[a]	2 : 3	0.2	2.5[g]	(2.0–3.0)	15.0 ± 10.6	14.2	(12–17)
PG[a]/CL[a]	2 : 5	0.2	2.5[g]	(2.0–3.0)	11.0 ± 11.4	13.8	(13–14)
CL[a]/PGL[a]	5 : 3	0.2	2.8[g]	(2.5–3.0)	15.8 ± 9.8	14.4	(12–17)
PG[a]/PE[a]	2 : 7	0.2	1.5	(0.5–2.0)	7.2 ± 3.1	13.8	(12–17)
PG[a]	–	0.2	0.8[h]	(0–2.0)	2.0 ± 1.5[h]	12.8[h]	(12–15)
PGL[a]	–	0.2	0.2[h]	(0–1.0)	0 ± 0.4[h]	12.6[h]	(11–15)
CL[a]	–	0.2	1.4	(0.5–3.0)	14.0 ± 4.5	14.3	(12–16)
PE[a]	–	0.2	1.0[h]	(0–2.0)	3.0 ± 2.0[h]	13.4[h]	(11–16)
CL[b]/PG[c]	1 : 1	0.2	1.9[g]	(0.5–3.0)	9.8 ± 6.9	15.4[g]	(14–18)
CL[b]/PS[b]	1 : 1	0.2	2.2	(2.0–3.0)	9.9 ± 3.0	16.1	(14–20)
CL[b]/PI[d]	1 : 1	0.2	2.1	(1.0–3.0)	9.0 ± 3.6	17.2	(16–20)
CL[e]/PG[c]	1 : 1	0.2	1.5	(0–2.5)	7.4 ± 4.3	16.2	(14–18)
CL[e]/PC[f]	1 : 1	0.2	1.2	(0–2.0)	6.8 ± 4.1		
CL[b]	–	1.0	1.3	(0–2.0)	8.4 ± 1.6	14.5	(12–17)
CL[b]	–	0.2	0.9	(1.0–2.0)	5.5 ± 4.4	13.5[h]	(13–15)
Wax D	–	0.2	2.6	(2.5–3.0)	15.1 ± 7.7	16.2	(14–19)
–	–	–	0.5	(0–2.0)	4.1 ± 3.8	13.0	(10–14)

[a]From *P. halosaccharolytica*
[b]From bovine brain
[c]Dioleoyl PG
[d]From porcine liver
[e]Hydrogenated Cl from bovine brain
[f]Dipalmitoyl PC
[g]Significant difference between the value and that of a negative control ($P < 0.05$)
[h]No significant difference between the value and that of a negative control ($P < 0.05$)

Abbreviations: PG, phosphatidylglycerol; PGL, glucosylphosphatidylglycerol; CL, diphosphatidylglycerol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine

antibody level (table 1), even when smaller amounts of phospholipid (50 μg) were used. Mixtures of two or more kinds of negatively charged phospholipids were active, while the single species of phospholipids or the mixture of a negatively charged phospholipid (phosphatidylglycerol) and a neutral phospholipid (phosphatidylethanolamine) were without effect.

Major fatty acids of phospholipids from *P. halosaccharolytica* are $C_{16:0}$ (42.8%), C_{17} (6.5%) and C_{19} (39.2%) cyclopropanoic acid [15], while those of diphosphatidylglycerol, and phosphatidylserine from bovine brain, and phosphatidylinositol from porcine liver were $C_{16:0}$ (8.6%), $C_{18:1}$ (11.9%) and $C_{18:2}$ (61%); $C_{18:0}$ (53.3%) and $C_{18:1}$ (40.6%); $C_{18:0}$ (48.3%) $C_{18:1}$ (13.1%), $C_{18:2}$ (6.5%) and $C_{20:4}$ (26.5%), respectively. Mixtures of two kinds of the animal phospholipids were also an active adjuvant, while the diphosphatidylglycerol alone was less active as in the case of bacterial phospholipids. Hydrogenated diphosphatidylglycerol [major fatty acids, $C_{16:0}$ (12.6%)

and $C_{18:0}$ (71%)] was less active in the induction of delayed hypersensitivity, whereas it still enhanced antibody production slightly. A mixture of synthetic dipalmitoyl phosphatidylcholine and the hydrogenated diphosphatidylglycerol reduced adjuvant activity for delayed hypersensitivity.

To elicit delayed hypersensitivity in random-bred Hartley guinea pigs, co-existence of at least two kinds of negatively charged phospholipids (diacyl form) was necessary. It was also indicated that the number of carbon atoms, the degree of unsaturation, branching and cyclopropane rings in the acyl moiety of negatively charged phospholipids may affect the adjuvant activity, in relation to the rigidity of lipid vesicles.

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