A SYNTHETIC ACYL POLYSACCHARIDE AND THE HEMAGGLUTINATION ACTIVITY

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Most bacterial lipopolysaccharides are highly active in the hemagglutination (HA) reaction. In fact, they are easily adsorbed onto the erythrocyte surface, thus sensitizing the cells, which in turn agglutinate in the presence of corresponding antiserum.

In 1948, Middlebrook and Dubos (1) reported that a mycobacterial component of polysaccharide nature could sensitize erythrocytes and in the presence of antiserum, HA took place. However, the chemical nature of the substance responsible for the HA reaction had remained obscure until the present authors and collaborators showed in 1959 and 1960 (2–4) that the antigen was a lipopolysaccharide never reported before. According to the results reported in those papers, the antigen isolated from bovine type mycobacteria (BCG) was a macromolecular lipopolysaccharide (LPS) composed of arabinose, mannose, and glucose in a small quantity, and fatty acid (22.8 per cent). The lipopolysaccharide from virulent human type mycobacteria (*Aoyama B*) had a closely related composition: arabinose, mannose, and fatty acid (18.7 per cent). The fact that HA antigens contain a certain amount of fatty acid is also true for a number of lipopolysaccharides of Gram-negative bacteria, having HA activities (5). It was therefore conceivable that the lipid moiety of the antigen was essential to HA activity.

In the course of the isolation of the lipopolysaccharide, the authors obtained several kinds of polysaccharide, one of which was polyarabinomannose (PS), composed of arabinose, mannose, and a trace of glucose. This polysaccharide did not contain lipid and was not active in HA reaction. But it precipitated with anti-mycobacteria serum and inhibited HA reaction. This indicated that PS had serologically specific site(s) but lacked some feature essential to sensitize erythrocytes.

An essential difference between the HA antigen LPS and PS was the fatty acid content. The authors tried therefore to synthesize chemically palmitoyl-polyarabinomannose(Pal-PS) and examined whether the product was active in HA reaction. Such a synthetic model could contribute to the elucidation of the mechanism of HA reaction.

In this paper, it will be reported that the presence of fatty acid in a serologically active polysaccharide molecule is an indispensable feature of HA antigen.

Materials and Methods

Chemical Analyses.—Hexose and pentose were determined by the anthrone (6) method using glucose as a standard and by the orcinol method (7) using arabinose, respectively. Phosphorus was measured colorimetrically (8) and nitrogen content by the elemental analysis (Dumas). For the detection of component sugars, a sample was hydrolyzed with 2 N H_2SO_4 at 100°C for 4 hours and was paper-chromatographed on Toyo filter paper No. 53 with acetone-butanol-water (7:5:2) and aniline hydrogen phthalate spray. For the estimation of lipid content, a sample was hydrolyzed with 3 N HCl at 100°C for 5 hours. After the hydrolysis, liberated fatty acid was extracted continuously with ether for 10 hours. The ether extract was washed with water, and it was concentrated to dryness on KOH pellets *in vacuo*. The residue was dissolved in methanol and was titrated with $\frac{1}{100}$ N NaOH. The lipid content was calculated as palmitic acid. In the case of the natural LPS, the lipid content was calculated from the dry weight of the fatty acid fraction. Infrared spectrum was measured as KBr pellet with Hitachi EPI-2 infrared spectrophotometer.

Serological Methods.—The procedure of HA reaction followed principally the method of Sorkin and Boyden (13) as reported previously (13). The sensitizing activity of a sample was expressed as the least amount of the antigen required to sensitize 1 ml of 2 per cent human O type erythrocyte suspension. Anti-*Aoyama B* rabbit serum, kindly supplied by the National Institute of Health, Tokyo, was used in a 50-fold dilution with saline throughout this experiment.

For the absorption of anti-*Aoyama B* rabbit serum with the natural LPS, the procedure was carried out as follows. One-half ml of the antiserum was incubated at 37°C for 2 hours with 300 μg of the natural LPS isolated from BCG. After the precipitate formed was centrifuged off, the supernatant solution was incubated again with 0.5 ml of washed human O type erythrocyte at 37°C for 1 hour with frequent shakings to remove the remaining LPS by adsorbing onto the cells. The cells were removed by centrifugation. The resulting absorbed antiserum was no longer active in agglutinating erythrocyte sensitized with the natural LPS.

For hemagglutination inhibition test with cholesterol, a modification of the HA procedure was used. One-half ml of serial saline dilution (2-fold) of cholesterol in the range of 250 to 4 μg/ml was mixed with 0.5 ml of a test antigen solution (5 μg/ml of saline) and was incubated at 37°C for 1 hour with occasional gentle shakings. After the incubation, 1 ml of 2 per cent erythrocyte suspension was added to the tubes. Keeping them at 37°C for 1 hour, the erythrocytes were separated by centrifugation, washed twice with saline, and finally suspended in a 1 per cent erythrocyte suspension. Three-tenths ml of antiserum of 1/50 dilution was mixed with 0.15 ml of sensitized erythrocyte suspension, and the mixture was incubated at 37°C for 1 hour. Tubes were kept in the cold room overnight and the result was read at the room temperature.

PS and Antigenic LPS.—Details of the isolation from defatted tubercle bacilli were described in the previous papers (2, 3). A crude polysaccharide fraction was extracted with borate buffer at pH 9 from tubercle bacilli which had been defatted with organic solvents and thereafter treated with 90 per cent phenol at 37°C. The polysaccharide mixture was further fractionated by means of starch zone electrophoresis into at least four kinds of polysaccharides. One of them was a mixture of PS and LPS. Both components were separated from each other by ultracentrifugation, 100,000 *g* for 6 hours. LPS was obtained from the pellet. PS was obtained from the supernatant by lyophilization. Both materials were purified by repeating the procedures.

EXPERIMENTAL

Purification of PS by Gel Filtration, Sephadex G-200.—PS had been separated from LPS by ultracentrifugation but still contained a small amount of LPS. Therefore, the further purifi-

cation was carried out by passing through a sephadex G-200 column. Sephadex G-200 was poured as a slurry into columns of 1 x 13 cm in an analytical scale and of 2.5 x 30 cm for the preparative use. They were washed with water. The amount of PS loaded on the column was about 1 to 2 mg dissolved in 0.5 ml of distilled water for analysis. For the preparation of components, about 100 mg of PS was applied to the column. The effluent was collected in 1 or 5 ml fractions, each of which was analyzed by the anthrone reaction. One of the typical patterns is shown in Fig. 1.

Preparation of Pal-PS.—Three 10 mg batches of the purified PS were dissolved in 1 ml of anhydrous pyridine, and to each solution various amounts of palmitoyl chloride, 5, 10, and 45 mg, were added. Test tubes were immediately sealed, shaken vigorously, and kept for 15

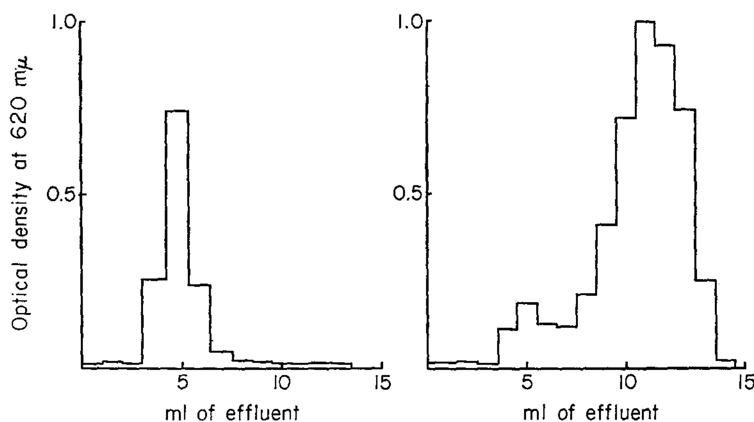

FIG. 1. One to 2 mg of LPS (left) or PS (right) was loaded on sephadex G-200 column, 1 x 13 cm. The elution was made with water, and each 1 ml of effluent was analyzed by anthrone reaction.

to 18 hours at room temperature with occasional shakings. After the termination of the reaction six volumes of absolute alcohol were added to each reaction mixture. The precipitates formed were separated by centrifugation and washed with ethanol at least three times to remove excess fatty acid, followed by washing with ether in the same way. After drying, yields of the acyl polysaccharides were in the range of 70 to 90 per cent to the original polysaccharide.

RESULTS

Chemical and Serological Properties of the Purified PS and the Natural LPS.—As shown in Fig. 1, LPS gave a single pattern with sephadex G-200 at the position of a non-diffusable material. This agreed with previous results which showed that LPS was a macromolecular substance (22.2S). On the other hand, PS was separated into two components by the treatment. The first peak represented only 8 per cent of the starting material and was found at the same position as that of LPS. As HA activity of the lyophilized substance of the first peak was 0.5 μg, it was most likely to be LPS. The second peak, however, was a major component (92 per cent), which was recovered by lyophilization. The HA activity was 32 to 64 μg, being markedly lower than that of the original

PS (8 μg). It was evidently due to the removal of the active LPS, which had contaminated the starting material. Chemical analyses of the major component indicated 44.4 per cent of hexose and 42.2 per cent of pentose. The sugar constituents so far detected were mannose and arabinose as well as a trace of glucose by paper chromatography. On acid hydrolysis, fatty acid was not found, in contrast to the fact that 22.8 per cent of fatty acid fraction by weight

TABLE I

Chemical Analyses and HA Activities

Material	Hexose	Pentose	Fatty acid	N	P	HA
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*μg*
LPS.	39.2	20.6	22.8	1.8	0.35	0.5
PS treated with sephadex G-200.	44.4	42.2	—	0.52	0.01	32–64

Hexose content: anthrone reaction as glucose reference. Pentose content: orcinol reaction as arabinose reference. Fatty acid content: see text. HA: The minimum amount of material required to sensitize 1 ml of 2 per cent erythrocyte suspension.

TABLE II

Synthetic Palmitoyl-Polyarabinomannose

Material	Palmitoyl-chloride, mg/10 mg PS	Yield	Palmitic* acid content	Molar ratio: fatty acid / monosaccharide
		mg	*per cent*	
Pal-PS-1	5	7.0	15.1	1:8.7
Pal-PS-2	10	8.6	24.7	1:4.7
Pal-PS-3	45	9.1	51.7	1:1.5

* Calculated from alkali titration of palmitic acid liberated from Pal-PS with HCl hydrolysis.

was yielded from the hydrolyzate of LPS. The sugar moiety of LPS consisted of mannose and arabinose as well as a trace of glucose, the same constituents of PS.

Synthetic Pal-PS.—The partial acylation of the purified PS was carried out in dry pyridine with palmitoyl chloride at the room temperature in a sealed tube. The extent of esterification evidently varied with the amount of palmitoyl chloride used, as shown in Table II. With 10 mg of PS and 5 mg of palmitoyl chloride, the product Pal-PS-1 contained 15.1 per cent of esterified fatty acid and the molar ratio of palmitic acid to average monosaccharide unit was 1:8.7. It was still readily soluble in water as well as in saline but Pal-PS-3 which contained 51.7 per cent of palmitic acid could not give a homogeneous

solution in water; some insoluble matter remained in an opalescent solution. Intermediate solubility was observed with Pal-PS-2.

Infrared spectrum of Pal-PS-1 in Fig. 2 showed absorption at 1720 cm^{-1}, characteristic of ester linkage; the intensity was comparable to that of the natural LPS. No absorption or shoulder at this region was noticed on the spectrum of PS, the starting material. The absorption at 850 cm^{-1} of Pal-PS-1 could not be assigned, but the mode of linkage between fatty acid and polysaccharide in Pal-PS-1 did not seem to be identical with that of the natural LPS.

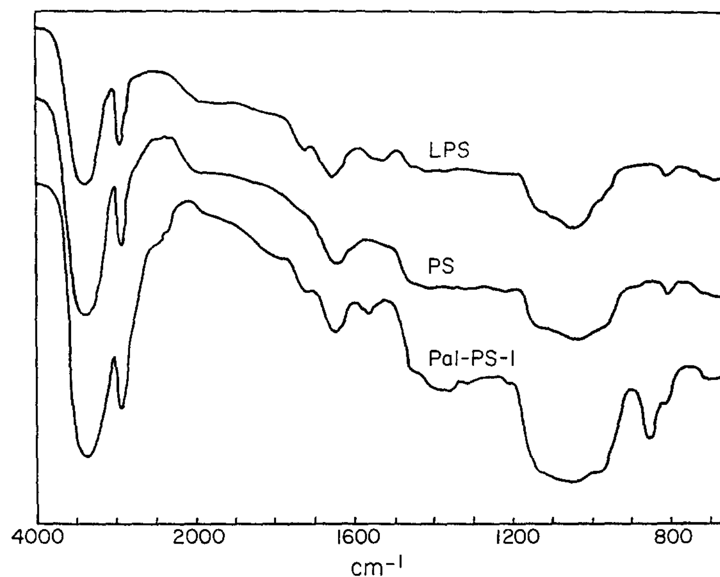

FIG. 2. Infrared spectra. LPS, the natural lipopolysaccharide of BCG; PS, the purified polyarabinomannose of BCG; Pal-PS-1, the synthetic palmitoyl-polyarabinomannose.

HA Activities of the Natural and Synthetic Lipopolysaccharides.—As shown in Table III, HA activities of various synthetic Pal-PS increased to about thirty to one hundred times over that of the original polysaccharide, PS. With Pal-PS-1, the activity was almost equivalent to that of LPS, that is, 0.5 μg of Pal-PS-1 was able to sensitize 1 ml of 2 per cent erythrocyte suspension. Pal-PS-2 and Pal-PS-3 also gave high activities. The difference in their activities could be due to solubility in physiological saline. So far as HA activity was concerned, it was therefore evident that the acylation could render the HA-inactive polysaccharide antigenic.

Antibody Involved in HA Reaction with Synthetic Pal-PS-1.—Although both natural and synthetic lipopolysaccharides exerted HA activity to the same extent, it was not known whether antibody involved in HA reaction was the

same. The anti-*Aoyama B* rabbit serum was absorbed with the natural LPS as described in the above section, to remove specific antibody to LPS. The results showed that the absorbed antiserum could not agglutinate erythrocytes sensitized with enough Pal-PS-1 as seen in Table IV. It appears, therefore, that the same kind of antibody was involved in HA reaction with both the natural and synthetic lipopolysaccharides.

TABLE III

HA Activities of Synthetic Lipopolysaccharides

Material	μg of material/ml for sensitization									
	128	64	32	16	8	4	2	1	0.5	0.25
Pal-PS-1	+++*	+++	+++	+++	+++	++	++	+	+	−
Pal-PS-2	+++	+++	+++	+++	+++	++	+	+	−	−
Pal-PS-3	+++	+++	++	++	+	+	+	−	−	−
PS	++	+	±	−	−	−	−	−	−	−
LPS	+++	+++	+++	+++	+++	++	++	+	+	−

* The pattern of settled erythrocytes was graded as +++ (complete hemagglutination) to − (no hemagglutination).

TABLE IV

HA Reaction with LPS-Absorbed Antiserum and Pal-PS-1 Sensitized Erythrocytes

Antiserum*	μg of Pal-PS-1 for sensitization				
	32	16	8	4	0
Not absorbed	++‡	++	++	++	−
Absorbed with LPS	−	−	−	−	−

* Anti-*Aoyama B* rabbit serum, in one-fiftieth dilution.
‡ Read as in Table III.

Inhibition of the HA Reaction with Cholesterol.—It appeared that the sensitization of erythrocytes involved a cell-antigen complex, stable on washing, and consequently that some constituent of erythrocytes combined with the antigen. As shown in Table V, the preincubation of Pal-PS-1 with various amounts of cholesterol abolished the sensitizing activity of Pal-PS-1. In fact, 5 μg of Pal-PS-1, ten times as much as the minimum required for HA activity, was completely inactivated with 63 μg of cholesterol. Pal-PS-1 once combined with cholesterol could not be adsorbed onto erythrocyte surface. It appeared that cholesterol moiety of erythrocyte was actually one of the adsorption sites of Pal-PS-1, the synthetic HA antigen.

DISCUSSION

The hemagglutination reaction involves at least two steps. First, an HA-antigenic substance must be adsorbed on the surface of erythrocyte even in a highly diluted solution to make the erythrocyte-antigen complex. Secondly, the erythrocyte-antigen complex agglutinates in the presence of corresponding antiserum. Therefore, HA-active substance must have at least both serologically specific site(s) and adsorption site(s) on the molecule. In this respect, it appears that the mycobacterial LPS had both sites, but the PS used in this experiment had only serologically specific site(s) and lacked adsorption site(s).

Taking into account that Pal-PS-1 showed a degree of HA activity com-

TABLE V

*Inhibition of Sensitizing Activity of Pal-PS-1 with Cholesterol**

Cholesterol, $\mu g/ml$	250	125	63	31	16	8	4	0	0
Pal-PS-1, $\mu g/ml$	5	5	5	5	5	5	5	5	0
Incubation at 37°C for 1 hour									
Erythrocyte suspension‡	1	1	1	1	1	1	1	1	1
Incubation at 37°C for 1 hour followed by washing of the treated erythrocytes									
Antiserum 1/50 dil., *ml*	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Hemagglutination	−	−	−	+	+	+	+	+	−

* The procedure is described in Serological Methods.

‡ Two per cent suspension in ml.

parable to that of the natural LPS, it seems reasonable that the acylation introduced a new feature; *i.e.*, adsorption site(s) to PS. According to the model experiment, the fatty acid moiety would constitute the adsorption site(s) in the case of the natural LPS. It is of great interest that adsorption onto erythrocytes would take place through the hydrophobic group of the antigen. The fact that cholesterol inhibited the adsorption of the antigen indicates that erythrocyte cholesterol could be one of the sites to which the hydrophobic group of the antigen was adsorbed and fixed. It was reported by Lüderitz *et al.* (9) that lipopolysaccharides of Gram-negative bacteria could form complex aggregates with cholesterol. At any rate, cholesterol in erythrocytes seems to have a significance in the process of sensitization.

The partial acylation of the polysaccharide was carried out with palmitoyl chloride and, since the infrared spectrum showed the presence of ester linkage, it is reasonable to consider that the polysaccharide was partially esterified. The ratio of palmitoyl group to monosaccharide unit was 1:8.7. In addition, it was

reported by Kabat (10) that a determinant unit of a polysaccharide was not more than hexasaccharide. Therefore, serologically specific sites of the polysaccharide do not seem to be significantly blocked by the acylation.

As reported by Boyden (11), erythrocytes carefully treated with tannic acid are able to adsorb a wider range of substances than untreated erythrocytes. The same effect was also obtained by treatment of erythrocytes with proteolytic enzymes (12). It is probable that the polar groups newly formed on the cell surface by the treatment help in anchoring materials. While these reactions depend on the modification of the erythrocyte surface, the results reported here give another method to produce HA activity.

Further work will be necessary to show whether the acylation procedure may also be applied to materials other than polysaccharide in order to render them HA-antigenic.

SUMMARY

From *Mycobacterium tuberculosis*, a serologically specific polysaccharide was purified. It contained mannose, arabinose, and a trace of glucose. It was not active in hemagglutination (HA) reaction.

Partial acylation of the polysaccharide was carried out with palmitoylchloride, and the synthetic lipopolysaccharide contained 15.1 per cent of esterified palmitic acid.

The synthetic lipopolysaccharide had hemagglutination activity and 0.5 μg was able to sensitize 1 ml of 2 per cent erythrocyte suspension; it had the same activity as the natural antigenic lipopolysaccharide.

The same antibody in the antiserum was involved in the hemagglutination reaction with both the synthetic and natural lipopolysaccharides.

The sensitizing activity of the synthetic lipopolysaccharide was completely neutralized by cholesterol.

Model experiments showed that the lipid moiety of hemagglutination antigen was essential for the sensitization of erythrocytes. The mechanism of the hemagglutination reaction is discussed.

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