Isolation and Characterization of Antitumor Lipopolysaccharide from *Proteus mirabilis*[†]

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Systematic isolation of the cell constituents of *Proteus mirabilis* RMS-203 was performed to find out localization of antitumor principle only in the lipopolysaccharide (LPS) layer of the cell wall fraction.

LPS with strong antitumor activity was extracted from *P. mirabilis* RMS-203 by phenol-water method followed by purification on DEAE-Sephadex A-50 column chromatography.

The main components of purified LPS were galactose, hexosamine, 2-keto-deoxy-octonic acid (KDO), myristic acid, β -hydroxymyristic acid and α,ϵ -diaminopimelic acid.

The minimal effective dose of LPS against Ehrlich solid carcinoma in mice was 0.1~1.0 μ g/mouse. LD₅₀ in mice and pyrogenicity in rabbits were 28 mg/kg and 10⁻³-10⁻⁵ μ g/rabbit, respectively.

As previously reported,^{2,15)} the living cells of *Proteus mirabilis* RMS-203, isolated from the pus of a woman's hepatomegalia, gave oncolytic effect on tumor-bearing mouse without any harmful influence on the host.

Isolation of LPS with antitumor activity from the cells of gram-negative bacteria has been extensively studied by many workers, especially by Shear and his colleagues¹⁸⁾ on *Serratia marcescens*. Chemical studies on LPS from *Proteus mirabilis* were done by Kotelko *et al.*¹⁰⁾ but not in relation to its antitumor activity. The antitumor effect of LPS from *Proteus* was only carried out by Mizuno *et al.*¹⁴⁾ using *P. vulgaris* as a source of LPS.

The present investigation deals with a systematic fractionation of the cells of *P. mirabilis* RMS-203 in relation to its antitumor activity. Isolation and purification of LPS, and its chemical and biological characterization are also presented.

Improvement in biological activity of LPS by mutation of the wild type as well as by

chemical modification will be reported in another paper.

MATERIALS AND METHODS

Organism. *Proteus mirabilis* RMS-203 isolated from the pus of a woman's hepatomegalia was used for isolation of the fraction with antitumor activity.

Medium and culture condition. For cultivation of *P. mirabilis* RMS-203, a 600 liter tank containing 400 liter medium was used. The medium was consisted of Polypepton (Daigo Eiyokagaku, Osaka) 1%, meat extract 1%, NaCl 0.5% and silicone KS 66 0.004%, being adjusted to pH 7.25 and sterilized at 120°C for 30 min. The pH after sterilization was 7.55. The seed culture with 500 ml of the medium in a 2 liter flask was shaken on a rotary shaker (200 rpm) for 40 hr and added to the tank. Fermentation conditions were as follows: temperature 37°C, aeration 200 liter/min, inner pressure 0.5~1.0 kg/cm², and agitation 200 rpm. Silicone KS 66 was added appropriately for defoaming.

After cultivation for 24 hr, the cells were collected by Sharples centrifuge and washed twice with tap water. Total yield of the cells was 1.75 kg by wet weight. The cells were stocked in a freezer until use.

Systematic fractionation of the cells of *P. mirabilis* RMS-203. Systematic fractionation of the cells of *P. mirabilis* RMS-203 was done by the method of Martin and Frank.¹³⁾

[†] Chemical and Biological Studies on Bacterial Lipopolysaccharide with Antitumor Activity. Part I.

Isolation of LPS. Isolation of LPS from the strain RMS-203 was done by the phenol-water method of Westphal *et al.*²³⁾ The cells (1 kg, wet) were extracted twice with 45% aqueous phenol at 65~68°C for 30 min. The aqueous phase was separated by centrifugation (9,000 × *g*, 10 min), and to the supernatant was added a mixture of CH₃COOK (2%) and ethanol (1: 2, v/v). The precipitate formed was collected by centrifugation, dialyzed against tap water and distilled water successively, and lyophilized. The yield of crude LPS (P-1) was 20 g. Further purification of LPS was done by chromatography on DEAE-Sephadex A-50. DEAE-Sephadex A-50 equilibrated with 0.2 M NaCl in 0.01 M phosphate buffer at pH 7.5 was packed into the 2.5 × 45 cm column. Crude LPS (2 g/200 ml in the above buffer) was applied on the column, and LPS was eluted with the same buffer. LPS fraction (tube No. 5~41) was collected and purified by rechromatography on the same type of the column.

Analytical methods. Total hexose was determined by the anthrone method of Yemm and Willis²⁵⁾ using glucose as the standard. Glucose was determined by glucose oxidase after hydrolysis of LPS by heating it in 1 N HCl or 1.3 N H₂SO₄ at 100°C for 4 hr in evacuated and sealed tubes. Total hexosamine was determined by the method of Boas.³⁾ The quantitative analysis of glucosamine and galactosamine was done by the borate buffer method of Good and Bessman.⁷⁾ Glucuronic acid was determined by the thioglycollic acid mannose-H₂SO₄ reaction described by Dishe,⁴⁾ and galacturonic acid by the method of Kerr and Seraiarian⁹⁾ or of Kotelko *et al.*¹⁰⁾

Protein was determined by the Folin-phenol reagent method of Lowry *et al.*,¹²⁾ using crystalline bovine albumin as the standard.

Heptose was determined by the cysteine-H₂SO₄ reaction of Osborn¹⁶⁾ using L-glycero-D-mannoheptose as the standard.

Phosphorus was determined by the colorimetric method of Allen¹⁾ using KH₂PO₄ as the standard.

Amino acid analysis was made by thin-layer chromatography¹⁷⁾ and amino acid autoanalyzer, Hitachi type KLA-2.

The colorimetric reaction with thiobarbituric acid described by Weissbach and Hurwitz²¹⁾ or by Osborn¹⁶⁾ was employed to determine 2-keto-3-deoxy-octonic acid.

Lipid A was extracted with a mixture of CHCl₃:methanol (2:1) after 0.1 N HCl hydrolysis or 1% acetic acid hydrolysis of LPS.²²⁾ Lipid B was extracted with a mixture of CHCl₃:methanol (2:1) from LPS without treatment with acid. Fatty acids of both ester and amide forms were determined by the method of Haskins and others.^{5,8,19,20)} Fatty acid methyl esters of LPS were prepared by the method of Yamakawa and Ueta.²⁴⁾ One microliter of methylated

samples was injected into Shimadzu Gas Chromatograph GS-1C flame-ionization unit equipped with stainless steel column (3 mm × 180 cm) packed with 25% polydiethylene glycol succinate coated on Diastolid L. The column was maintained at 180°C and the injector port at 230°C.

LD₅₀ determination. Lethal toxicity of the isolated preparations against mouse was determined with ddY or RFVL mice (female, about 20 g) which were injected intravenously with the preparations dissolved in saline. Calculation of LD₅₀ was made according to Litchfield-Wilcoxon's method.¹¹⁾

Antitumor activity. Antitumor activity of the preparations from *P. mirabilis* RMS-203 was tested by observation of necrosis in tumor section or by the Pigment test.

Ehrlich ascites cell suspension (2 × 10⁶ cells/0.1 ml) was implanted subcutaneously in an area of right armpit of ddY or RFVL mice. Samples for testing antitumor activity were injected intravenously to the mice on 7 to 9 days after transplantation, when a detectable tumor was developed. One week after administration, the mice were sacrificed, and necrosis in tumor sections was examined. For the Pigment test, the modified method of Goldacre and Sylvén⁶⁾ was used. At 24 hr after administration of test samples, the mice were injected intravenously with 0.3 ml of 2% Light green SF yellowish or Pontamine sky blue 6B (Tokyo Kasei Kogyo Co., Ltd.) and sacrificed at 2 hr after injection of the pigment. The tumor tissue of non-treated animal was stained greenish or bluish with the pigment whereas that injected with an active LPS fraction was not.

The results obtained by observation of necrosis agreed with those obtained by the Pigment test. Therefore, the antitumor test was done mainly by the Pigment test for the sake of time saving.

Pyrogenicity. Pyrogenicity was determined in rabbits by standard procedures in The United States Pharmacopeia (1970).

RESULTS AND DISCUSSION

Systematic procedure for the fractionation of the cell constituents of *P. mirabilis* RMS-203 is shown in Chart 1. Similar to the results obtained by Martin and Frank¹³⁾ for the cells of *Escherichia coli* B, the cells of *P. mirabilis* RMS-203 was fractionated into cytoplasm and cell wall portion, and the latter was further into four main fractions, lipoprotein, LPS, protein granule and mucopoly-

CHART 1. Systematic Isolation of Cell Wall Fractions from *Proteus mirabilis* RMS-203.TABLE I. CHEMICAL COMPOSITION AND BIOLOGICAL PROPERTY OF EACH CELL WALL FRACTION ISOLATED FROM *Proteus mirabilis* RMS-203

	Hexose	Hexosamine	Heptose	Protein	Fatty acid	Antitumor activity ^{a)}
Whole cells	1.6%	0.4%	1.1%	58.5%	6.1%	positive
Cytoplasm	2.3	1.5	0.5	54.7	—	negative
Cell wall fraction						
Lipoprotein	0	0	0.4	86.5	12.3	negative
Lipopolysaccharide	8.7	9.5	2.2	2.0	31.2	positive
Protein granule	0	0	0	110	—	negative
Crude mucopolymer	4.3	9.0	1.4	37.3	22.9	negative
Mucopolymer	0.9	27.4	0.1	13.8	0	negative

^{a)} Antitumor activity of each fraction was tested by observation of necrosis in tumor section at one week or by the Pigment test at 24 hr after intravenous administration of the samples at a dose of 1 ~ 10 μ g per mouse.

mer. Three layers of the cell wall, lipoprotein, LPS, and protein granule and mucopolymer contribute 62.1%, 20.4% and 17.5%, respectively, to the total weight of the cell wall which were broadly similar to those values for *E. coli* B by Martin and Frank, 60%, 12% and 21%.

Chemical composition and antitumor activity of each fraction are given in Table I.

Successful separation of the cell-wall layers is recognized by the result of analysis of chemical composition of each layer, such as lipoprotein layer with lipid and protein, LPS with lipid, hexose, hexosamine and heptose, protein granule with protein alone, and mucopolymer with hexosamine and protein.

The test for antitumor activity of each layer clearly indicated localization of the

activity at LPS in the cell constituents.

Further purification of the antitumor principle was thus concentrated to LPS fraction of the cells of *P. mirabilis* RMS-203.

As was in most investigations in the past, LPS was prepared by the phenol-water extraction method by Westphal *et al.* as shown in Chart 2. Twenty grams of crude LPS (P-1) was obtained from 1 kg wet weight of the cells. The yield was 2%. Further purification of LPS by column chromatography on DEAE-Sephadex A-50 are given in Chart 3.

Anthrone-positive LPS fraction was eluted with 0.2 M NaCl in separation from the contaminated RNA fraction eluted with higher concentration of NaCl. The yield of purified LPS (P-3) was 6.4 g or 0.64% in this procedure.

CHART 2. Extraction and Purification of LPS from *Proteus mirabilis* RMS-203.

CHART 3. Purification of Crude LPS Extracted from *Proteus mirabilis* RMS-203 by DEAE-Sephadex A-50 Column Chromatography.

DEAE-Sephadex A-50 was equilibrated with 0.2 M NaCl in 0.01 M phosphate buffer (pH 7.5) and packed into the 2.5×45 cm column. Crude LPS (P-1, 2g) dissolved in the same buffer (200 ml) was applied on the column and eluted with the same buffer (10 ml in each tube). After elution of LPS, RNA fraction was eluted with the gradient concentration of NaCl (0.2→0.6 M) in the same buffer.

LPS P-1

LPS P-3

FIG 1. Tiselius Electrophoretic Patterns of LPS P-1 and P-3 Isolated from *Proteus mirabilis* RMS-203.

Condition: Hitachi Tiselius Electrophoretic Apparatus HTB-2A 0.1 M Veronal buffer, pH 8.6, 100 V, 7 mA, at 14.3°C.

The electrophoretic patterns of purified LPS and its chemical and biological properties are shown in Fig. 1 and Table II, respectively.

LPS (P-3) was found to be a single compound by means of electrophoretic analysis. Removal of RNA from LPS (P-1) was clearly

TABLE II. CHEMICAL COMPOSITION AND BIOLOGICAL PROPERTIES OF LPS
ISOLATED FROM *Proteus mirabilis* RMS-203

	LPS P-1 ^{a)} %	LPS P-3 ^{b)} %	LPS from 1959 ^{c)} %
[Elementary analysis]			
C	35.9	43.1	
H	6.32	7.36	
N	4.20	3.90	
P	3.45	1.73	
ash	—	7.93	
[UV absorption at 260 nm]			
$E_{1\text{cm}}^{1\%}$	47.3	7.1	
RNA	16.1	0	
[Chemical composition]			
Total hexose	12.3	17.6	6.2
glucose	2.0	1.9	6.2
galactose	10.3	15.7	0
Heptose	5.7	5.8	12.6
Total hexosamine	9.8	13.9	13.4
glucosamine	5.5	5.8	6.0
galactosamine	4.3	8.1	7.4
2-Keto-3-deoxy-octonic acid (KDO)	1.4	1.6	3.8
Total uronic acid	—	5.4	13.7
glucuronic acid	(1.5)	(1.4)	trace
galacturonic acid	—	4.0	13.7
Protein	4.6	0.7	
Amino acid			
glycine	1.1	0	
α,ϵ -diaminopimelic acid (DAP)	10.3	8.9	
Lipid A (CH ₃ COOH)	—	33.3	
(HCl)	26.9	16.6	
Lipid B	2.7	1.7	
Total fatty acid (GLC)	7.6	7.4	
(NH ₂ OH)	7.6	7.3	
ester-linked	4.9	6.2	
amide-linked	2.7	1.1	
myristate	3.8	3.6	
15 anteiso	0.2	0.2	
palmitate	0.6	0.5	
palmitoleate	0.2	0.1	
β -OH myristate	2.6	2.8	
Sugars detected by PPC, TLC and GLC	Ribose		
	Glucose	Glucose	Glucose
	Galactose	Galactose	
	Heptose	Heptose	Heptose
	KDO	KDO	KDO
	Galacturonic acid	Galacturonic acid	Galacturonic acid
	Glucosamine	Glucosamine	Glucosamine
	Galactosamine	Galactosamine	Galactosamine
Amino acids found on TLC and amino acid autoanalyzer	Glycine	Glycine	
	Alanine	Alanine	
	Lysine	Lysine	Lysine
	Leucine	Leucine	
	Glutamic acid	Glutamic acid	
	DAP	DAP	
	(Aspartic acid)	(Aspartic acid)	
	Ethanolamine	Ethanolamine	Ethanolamine
[Biological properties]			
Antitumor activity $\mu\text{g}/\text{mouth}$	0.1 ~ 1.0	0.1 ~ 1.0	
Toxicity LD ₅₀ (mouse, IV) mg/kg	48	30	
Pyrogenicity $\mu\text{g}/\text{rabbit}$	10 ⁻³ ~ 10 ⁻⁷	10 ⁻⁹	

^{a)} LPS P-1: Crude LPS isolated by phenol-water method followed by precipitation with CH₃COOK and ethanol as described in Chart 2.

^{b)} LPS P-3: Purified LPS obtained by DEAE-Sephadex chromatography of LPS P-1 as described in Chart 2.

^{c)} LPS from 1959: LPS isolated from *Proteus mirabilis* 1959. The data cited from the paper by Kotelko *et al.*¹⁰⁾

demonstrated by loss of UV-absorption at 260 nm in LPS (P-3).

Galactose rather than glucose is a major hexose constituent of the LPS of *P. mirabilis* RMS-203 in contrast to the result obtained by Kotelko *et al.*¹⁰⁾ of *P. mirabilis* strain 1959 in which only glucose was detected as a hexose constituent of LPS.

The differences between above two strains of *P. mirabilis* were also demonstrated in the amounts of heptose and galacturonic acid found in the constituents of their LPS. A part of heptose, KDO and galacturonic acid constituents of the strain 1959 might be replaced by galactose in the strain RMS-203. Hexosamine content was, however, in good agreement with both strains suggesting the resemblance of their lipid A fraction composed of hexosamine polymer.

Most of fatty-acid constituents in LPS is ester-linked one mainly composed of myristic and β -OH myristic acids.

Lysine is a sole amino acid detected in LPS of strain 1959, but the presence of some other amino acids is also detected in the case of strain RMS-203.

Antitumor effect of LPS from *P. mirabilis* RMS-203 on the solid-type of Ehrlich carcinoma appeared after single intravenous injection even at a concentration as low as 0.1 to 1 mg/kg (2 to 20 μ g/mouse, once). A similar anti-tumor effect but with higher concentration (100 μ g/mouse, 3 times) was reported by Mizuno *et al.*¹⁴⁾ using LPS of *P. vulgaris* OX 19 origin. A comparable degree of toxicity was noted for LPS from *P. mirabilis* RMS-203 with that for *P. vulgaris* OX-19 (LD_{50} =80 mg/kg, mouse, ip).

In conclusion, the existence of toxicity and pyrogenicity in the present LPS prevents its therapeutic use in spite of its significant anti-tumor activity.

Further studies in an attempt to decrease these unfavorable features of LPS by microbiological and chemical modification will be

reported in the next paper.

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REFERENCES

- 1) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1960).
- 2) M. Arakawa, K. Sugiura, H. C. Reilly and C. C. Stock, *Gann*, **59**, 117 (1968).
- 3) N. Boas, *J. Biol. Chem.*, **204**, 553 (1953).
- 4) Z. Dishe, *ibid.*, **171**, 725 (1947).
- 5) R. F. Godden, N. H. LeBlanc and C. M. Wright, *Analyt. Chem.*, **27**, 1251 (1955).
- 6) R. J. Goldacre and B. Sylvén *Brit. J. Cancer*, **16**, 306 (1962).
- 7) T. A. Good and S. P. Bessman, *Analyt. Biochem.*, **9**, 253 (1964).
- 8) W. T. Haskins, *Analyt. Chem.*, **33**, 1445 (1961).
- 9) S. E. Kerr and K. Seraidarian, *J. Biol. Chem.*, **159**, 211 (1945).
- 10) K. Kotelko, O. Lüderitz and O. Westphal, *Biochem. Z.*, **343**, 227 (1965).
- 11) J. T. Litchfield and F. Wilcoxon, *J. Pharmacol. Exp. Therap.*, **96**, 99 (1949).
- 12) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 13) H. H. Martin and H. Frank, *Z. Naturforsch.*, **17b**, 190 (1962).
- 14) D. Mizuno, O. Yoshioka, M. Akamatsu and T. Kataoka, *Cancer Res.*, **28**, 1531 (1968).
- 15) T. Murata, M. Arakawa, Y. Sugiya, Y. Inazu, Z. Hattori, Y. Suzuki, H. Minakami, M. Nakahara and H. Okazaki, *Life Sci.*, **4**, 1055 (1965).
- 16) M. J. Osborn, *Proc. Nat. Acad. Sci.*, **50**, 499 (1963).
- 17) G. Pataki, *J. Chromatog.*, **17**, 580 (1965).
- 18) M. J. Shear, F. C. Turner, A. Perrault and T. Shovelton, *J. Nat. Cancer Inst.*, **4**, 81 (1943).
- 19) F. Synder and N. Stephens, *Biochem. Biophys. Acta*, **34**, 244 (1959).
- 20) H. Tauber, *Federation Proc.*, **19**, 245 (1960).
- 21) A. Weissbach and J. Hurwitz, *J. Biol. Chem.*, **234**, 705 (1959).
- 22) O. Westphal and O. Lüderitz, *Angew. Chem.*, **66**, 407 (1954).
- 23) O. Westphal, O. Lüderitz and F. Biester, *Z. Naturforsch.*, **7b**, 148 (1952).
- 24) T. Yamakawa and N. Ueta, *Japan J. Exp. Med.*, **34**, 361 (1964).
- 25) E. W. Yemm and A. J. Willis, *Biochem. J.*, **57**, 508 (1954).