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Mitogenicity and Adjuvanticity of Lipopolysaccharide from a Marine Bacterium, *Vibrio anguillarum*, in Mice

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The effects of lipopolysaccharides (LPS) extracted from three O-serotype (A, B, and C) strains of *Vibrio anguillarum* on the murine immune response were studied. Although the LPSs lack 2-keto-3-deoxyoctonate (KDO) and the sugar compositions of their core regions are markedly different, depending on the O-serotype, they were all able to induce a mitogenic effect on *in vitro*-cultured spleen cells of C57BL/6 mice. The LPS was capable of increasing the incorporation of ^{3}H-thymidine into spleen cells that had been treated with rabbit antithymocyte serum in the presence of complement in order to kill T-lymphocytes. When sheep erythrocytes and LPS were injected intraperitoneally into BALB/c mice, the LPSs exhibited an enhancing effect on the antibody response regardless of the serotype.

These findings suggest that these biological effects of *V. anguillarum* LPSs from different O-serotype strains are little affected by the absence of KDO and the differences in sugar composition.

Keywords——*Vibrio anguillarum*; lipopolysaccharide; 2-keto-3-deoxyoctonate; mitogenicity; adjuvanticity

Vibrio anguillarum, a gram-negative and halophilic marine bacterium, causes vibriosis in fish, both wild and cultured;[1] this is one of the most serious diseases affecting various kinds of fish. We previously reported that the whole cell of *V. anguillarum* has significant adjuvant and mitogenic effects on splenocytes, and a stimulating effect on the reticuloendothelial system.[2] Recently, we found that the lipopolysaccharide (LPS) of the organism lacks 2-keto-3-deoxyoctonate (KDO) and several kinds of neutral sugars in its core region, depending on the O-serotype (A, B, or C).[3]

In this paper, we describe the relationship between the sugar composition and the immunostimulating effects (mitogenicity and adjuvanticity) of *V. anguillarum* LPS in mice.

Materials and Methods

Animals——Both male and female C57BL/6Cr and BALB/c mice, 10—15 weeks old, were used in the experiments. These mice were supplied by our animal colony.

Bacterial Strains——The following strains of different O-serotypes of *V. anguillarum* were used: type A (PT-24, TA-3, H-47, H-107, H-124); type B (PT-514, H-60, H-88); type C (PT-213, H-102); type unknown, H-137. One strain (PT-514) was kindly provided by Drs. T. Kitao and T. Aoki, and strains PT-24 and PT-213 were gifts from Drs. K. Muroga and T. Sasaki, respectively. The other strains were isolated by us from cultured ayu (*Plecoglossus altivelis*) at Lake Hamana and identified on the basis of their biochemical characteristics and by O-agglutination testing with antisera that were kindly supplied by Dr. T. Sasaki.[4]

These organisms were cultured aerobically at 27 °C for 16 h in nutrient broth containing 1% NaCl. The cells were harvested by centrifugation (10000 × *g*, 20 min) at 4 °C, washed twice with saline and dried with acetone.

Preparation of LPS——LPS was isolated from acetone-dried cells by the phenol–water technique of Westphal *et*

TABLE I. Sugar Compositions of Lipopolysaccharides (LPSs) from Strains of *Vibrio anguillarum*

Serotype[a]	Glucose	Galactose	Fructose	Rhamnose	Fucose	Ribose	Mannose	Hep L-D[b]	KDO[c]	Uronic acid	Glucosamine	Galactosamine	Mannosamine	Unknowm amino sugar[d]	
														P_1	P_2
A	+	+	−	−	−	−	−	+	−	−	+	−	−	−	−
B	+	−	+	−	−	−	−	+	−	−	+	−	−	−	+
C	+	−	−	+	−	−	−	+	−	−	+	+	−	−	−
Unknown	+	−	−	−	+	+	+	+	−	−	+	+	−	−	−

a) Strains used: Serotype A, PT-24, H-47, H-107, H-124; Serotype B, PT-514, H-60, H-88; Serotype C, PT-213, PB-1, H-102; Unknown, H-137.
b) Hep L-D, L-glycero-D-mannoheptose.
c) KDO, 2-keto-3-deoxyoctonate.
d) P_1 and P_2, unknown amino sugar present in *V. parahaemolyticus* LPS.

al.,[5] and purified by ultracentrifugation (105000×*g*, 3 h, 6 times) and treatment with ribonuclease (20 μg/ml in 25 mM Tris-HCl buffer, pH 7.4). The sugar compositions of LPSs extracted from different O-serotype strains are summarized in Table I.

Glucose, L-glycero-D-mannoheptose, and glucosamine were detected in all LPSs, whereas LPSs of serotypes A, B, and C are characterized by the presence of galactose, fructose, and rhamnose, respectively. LPS of unknown serotype contains fucose, ribose, and mannose. These results will be published in detail elsewhere (manuscript in preparation).

Mitogen and Adjuvant——Concanavalin A (Con A; Boehringer Mannheim, West Germany) was used as a specific T-lymphocyte mitogen.[6] The LPS, which was extracted from *Salmonella typhimurium* LT-2 by the hot phenol–water extraction method,[5] was used as an adjuvant and a specific B-lymphocyte mitogen.[7]

Mitogenic Studies——A suspension of spleen cells of C57BL/6 mice was prepared as previously described.[2] The splenocytes were suspended in RPMI-1640 medium (GIBCO, U.S.A.) supplemented with 10% fetal calf serum (GIBCO). One-tenth ml (5×10^5 cells) of the cell suspension and 0.1 ml of a suspension of *Vibrio* LPS were placed in a 96-well microplate (Falcon #3042, U.S.A.). The plate was incubated at 37 °C in an atmosphere of 5% CO_2 and 95% air. Each well was pulsed with 0.25 μCi of thymidine-6-^{3}H (^{3}H-TdR; Radiochemical Centre, England) for the final 16 h of incubation. After incubation for 64 h, the cultured spleen cells were harvested on a glass fiber filter (Whatman, GF/C) with an automatic cell harvester (Abe Scientific Co., Ltd., Chiba). The filters were washed with distilled water and dried. The radioactivity taken up by the cells was measured with a scintillation counter (Aloka, LSC-661, Aloka Co., Tokyo). The results are expressed as the mean counts per minute (cpm) per well with the standard error (S.E.).

Rabbit Antithymocyte Serum (ATS) Treatment of Spleen Cells——ATS was prepared according to the method of Gray *et al.*[8] The prepared ATS showed cytotoxicity titers of 2^9 and 2^3 for mouse thymocytes and bone marrow cells, respectively, based on the trypan blue dye exclusion method.[9] The spleen cells from C57BL/6 mice were incubated with suitably diluted ATS for 45 min at 37 °C in the presence of complement (guinea pig serum) in order to kill T-lymphocytes in the spleen cell population. As a reference, normal rabbit serum (NRS) was used.

Hemolytic Plaque Assay——Sheep red blood cells (SRBC) were used as the antigen. SRBC (10^8) and/or *Vibrio* LPS were injected intraperitoneally into BALB/c mice. Spleen cells were prepared from each mouse and the number of plaque-forming cells (PFC) in the spleen was determined by the technique of localized hemolysis in agar.[10] The results are expressed as mean PFC per four dishes with the S.E.

Results and Discussion

To determine the mitogenic effect of *Vibrio* LPS, various amounts (1—100 μg/ml) of LPS from strain H-124 were added to the cultured spleen cells of C57BL/6 mice and the incorporation of ^{3}H-TdR into the cells *in vitro* was measured.

As shown in Table II, doses of 5 and 10 μg/ml of the LPS were most effective on the incorporation of ^{3}H-TdR into the cultured spleen cells. The stimulation index was reduced at the concentration of 25 μg/ml or more of LPS. The results suggest that the LPS has a slight cytotoxic effect on the cultured cells at high concentrations.

TABLE II. Effect of Dose of *V. anguillarum* LPS on Mitogenicity for Cultured Spleen Cells of C57BL/6 Mice *in Vitro*

Stimulant	Dose (μg/ml)	^{3}H-TdR uptake	
		cpm ± S.E.[a]	Stimulation index[b]
V. anguillarum H-124 LPS	100	3542 ± 274	3.5
	50	3942 ± 322	3.9
	25	3451 ± 304	3.4
	10	4968 ± 121	4.9
	5	4249 ± 101	4.2
	1	1849 ± 168	1.8
S. typhimurium LPS	10	3672 ± 279	3.6
Concanavalin A	1	31023 ± 566	30.7
None (control)		1012 ± 69	

a) Assayed 64 h after incubation of spleen cells (5×10^5 cells in 0.2 ml of medium) *in vitro*.
b) Stimulation index = experimental cpm/control cpm.

TABLE III. Mitogenic Effect of *V. anguillarum* LPS on Cultured Spleen Cells of C57BL/6 Mice *in Vitro*

V. anguillarum LPS		Dose (μg/ml)	^{3}H-TdR uptake	
Serotype	Strain		cpm ± S.E.[a]	Stimulation index[b]
A	PT-24	10	11875 ± 967	12.8
	H-47	10	10686 ± 156	11.5
	H-107	10	9311 ± 107	9.5
	H-124	10	7038 ± 115	7.6
B	PT-514	10	14140 ± 446	15.2
	H-60	10	13832 ± 460	14.9
	H-88	10	3234 ± 112	3.5
C	PT-213	10	16637 ± 356	17.9
	H-102	10	6913 ± 163	7.4
Unknown	H-137	10	13247 ± 181	14.3
S. typhimurium LPS		10	8740 ± 818	9.4
Concanavalin A		1	57163 ± 298	61.5
None (control)			929 ± 118	

a) See footnote *a*) to Table II.
b) See footnote *b*) to Table II.

The mitogenic effects of LPSs from 10 strains having different O-serotypes were determined (Table III). When the LPSs were added to the cultured spleen cells at 10 and 100 μg/ml (data not shown), all LPSs showed a mitogenic effect, regardless of the O-serotype.

However, the reason why the potency of the mitogenic effects of these LPSs was variable is not clear. The carbohydrate and lipid contents of the LPSs were different from each other.[3] The difference of chemical properties may result in a marked difference in the potency of mitogenic activity of LPS.

Next, to determine whether the LPS is a T- or B-lymphocyte mitogen, T-lymphocytes in a splenocyte population were killed by ATS in the presence of complement (Table IV).

After the treatment with ATS, the mitogenic response of splenocytes to Con A was markedly reduced, whereas the incorporation of ^{3}H-TdR into ATS-treated spleen cells

Table IV. Mitogenic Effect of *V. anguillarum* LPS on B-Lymphocytes in Cultured Spleen Cells of C57BL/6 Mice *in Vitro*

Stimulant	Dose (μg/ml)	NRS-treated spleen cells		ATS-treated spleen cells[b]	
		cpm ± S.E.	Stimulation index[a]	cpm ± S.E.	Stimulation index
V. anguillarum TA-3, LPS	10	922 ± 50	2.9	1878 ± 242	2.3
S. typhimurium LPS	10	1221 ± 56	3.8	3444 ± 461	4.1
Concanavalin A	10	6057 ± 459	18.8	766 ± 97	0.9
None (control)	1	322 ± 69		833 ± 97	

a) See footnote *b*) to Table II.
b) Spleen cells were treated with rabbit antithymocyte serum in the presence of guinea pig complement.

Table V. Adjuvant Effect of *V. anguillarum* LPS on Anti-SRBC PFC Response in the Spleen of BALB/c Mice

Treatment[a]	Serotype	Average number of PFC per spleen (mean ± S.E.)[b]	Stimulation index[c]
V. anguillarum H-124	A	28100 ± 3769	18.7
H-88	B	37600 ± 2288	25.1
H-102	C	41400 ± 3782	27.6
S. typhimurium LPS		33600 ± 282	22.4
SRBC alone (control)		1500 ± 450	

a) Mice were injected *i.p.* with 10^8 SRBC and 100 μg of *Vibrio* LPS or 100 μg of *S. typhimurium* LPS on day 0.
b) Assayed 3 d after immunization. Four mice per group.
c) Stimulation index = PFC of experimental group/PFC of control group.

incubated with *S. typhimurium* LPS increased. The LPS of *V. anguillarum* TA-3 was also able to stimulate the incorporation of ^{3}H-TdR into ATS-treated spleen cells as well as NRS-treated spleen cells.

The adjuvant effect of *Vibrio* LPS on the antibody response to xenogeneic erythrocytes in BALB/c mice was determined (Table V). The LPSs from strains of all three serotypes, H-124 (type A), H-88 (type B), and H-102 (type C), exhibited a significant adjuvant effect similar to that of *S. typhimurium* LPS on day 3 after the immunization. However, there was no difference in the adjuvant effect among the LPSs having the three O-serotypes.

It was previously reported that the whole cell,[11] KDO-free LPS,[12] and the culture filtrate[13] of *V. anguillarum* have antitumor activity against Ehrlich carcinoma cells in mice. Furthermore, we demonstrated that the whole cells of *V. anguillarum* has a significant mitogenic effect on splenocytes, and an adjuvant effect on acid phosphatase activity in peritoneal exudate cells in mice.[2] Recently we detected a difference in cellular sugar composition of the organisms according to serotype, using gas-liquid chromatography.[14]

In this paper, we show that the purified LPS as well as the whole cell of *V. anguillarum* is capable of eliciting the mitogenic and adjuvant effects in mice. The LPS of *V. anguillarum* as well as the LPS of other species of *Vibrio*,[15] possesses no KDO, a regular sugar component of the core region of most gram-negative bacterial LPS, and the sugar composition of LPS in the different serotypes of *V. anguillarum* strains differed with the serotype (Table I). A few papers have reported that the polysaccharide moiety of LPS has a polyclonal activation and adjuvant effect on the antibody response *in vitro*.[16] However, it is generally considered that the

active principle of LPS is a lipid A region.[17] We conclude that KDO is unnecessary as an active principle for the immunostimulating effects of LPS, since we found that KDO-free LPS has mitogenic and adjuvant effects in mice, as do other gram-negative bacterial LPSs, which possess KDO.[18] We also showed that the immunostimulating effects of *V. anguillarum* LPSs from different serotype strains were little affected by the differences in their sugar compositions (Tables III and V). This finding indicates that the kind of sugar present in the polysaccharide moiety of LPS is not important in the expression of the biological activity of LPS.

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