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Cytotoxic Effect and Fatty Acid Composition of Lipopolysaccharide from *Vibrio vulnificus*

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Abstract

Lipopolysaccharide(LPS) from *Vibrio vulnificus* was purified, the fatty acid composition was analyzed, and *Limulus* gelation activity and lethal toxic activity were tested in order to investigate the cause of cytotoxicity by *V. vulnificus*. These results were compared to those of *Escherichia coli* LPS and *Salmonella typhimurium* LPS. LPS from *V. vulnificus* had a different fatty acid composition from those of *E. coli* and *S. typhimurium*. The major fatty acid from each LPS was lauric acid for *E. coli*, capric acid for *S. typhimurium*, and myristic acid for *V. vulnificus*. The *Limulus* gelation activities of three LPSs were the same(0.1ng/ml) and the lethal toxicity in BALB/c mouse of *V. vulnificus* LPS was similar to those of *E. coli* LPS and *S. typhimurium* LPS. Such factor as exotoxin need to be considered to be the cause of cytotoxicity by *V. vulnificus* LPS.

Key words : cytotoxic effect, fatty acid composition

Introduction

Bacterial cell wall lipopolysaccharide(LPS, endotoxin) is a prominent macromolecular component of the outer membrane of gram-negative bacteria[1]. LPS is known to be composed of three distinct regions. The outermost region is a polysaccharide chain with repeating oligosaccharide units of variable length and constitutes the O-antigen region. This is connected to the core polysaccharide chain. It is linked through one or more units of a unique 8-carbon sugar, 2-keto-3-deoxy-octonate (KDO), to a complex hydrophobic component, lipid A. Anchored in the outer membrane, lipid A is a derivative of glucosamine disaccharide[2-4]. LPS has numerous biological activities, many of which are apparently caused by the lipid A[5-7]. Lipid A is the primary agent for the endotoxicity and displays a number of biological functions. Substituents usually attached to it are long chain fatty acyl groups in both ester and amide linkage. Of the acyl residues in gram-negative bacteria, lauric, myristic, and palmitic acid are the major components. The endotoxicity of LPS depends on its fatty acid composition.

A basophilic bacterium, *Vibrio vulnificus* is the most invasive of all the vibrio species and is related to the high incidence of septicema which correlated with the high death rate[8]. *V. vulnificus* causes acute fulminatng wound infections and septicema in humans. The cause for the lethal infection by *V. vulnificus* was known little. Therefore, cell wall LPS as the cause for toxicity by *V. vulnificus*, *E. coli*, and *S. typhimurium* was selected, extracted LPSs and analyzed the fatty acid compositions of the LPSs, and then tested the *Limulus* gelation activities and lethal toxicities by LPSs in this study.

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Materials and Method

Bacterial strains and culture conditions

Cells of *Escherichia coli* ATCC 25922 and *Salmonella typhimurium* ATCC 14028 were grown with aeration in tryptic soy broth(TSB) at 37°C for 24h. A virulent *Vibrio vulnificus* P-1 was grown in TSB which contained additional 5g of NaCl. The above bacteria were obtained from the microbiology laboratory of the Paik Hospital in Pusan, Korea.

Extraction and purification of LPS

A partially modified procedure of the hot phenol-water method[9] was applied to extract LPS. The bacterial cells were separated from the growth medium by centrifugation and were suspended in distilled water, sonicated, and centrifuged. The supernatant was centrifuged and the resulting pellet was extracted with 100ml of 45% phenol. The mixture was shaken at 65-68°C for 20min, cooled in ice bath, and centrifuged. The upper aqueous phase was removed and was dialyzed against distilled water for 3 days. The dialyzed solution was freeze-dried.

Fatty acid analysis of LPS

Fatty acids were analyzed as their methyl esters. Total fatty acids released from LPS by 2N HCl, were converted to methyl esters by incubating in 10% BF_3-MeOH at 70°C for 10min in sealed tube. The solution was allowed to cool and n-hexane was added with mixing to extract fatty acid. The esters were separated in a 3mm x 3m glass column packed with 10% 1,4-butanediol succinate on Chromosorb W(60 - 80 mesh) at 185°C by Gas Chromatograph. Ten kinds of fatty acids in Table 1 were used as the standard ones.

Limulus lysate assay

Equal volumes of LPS and *Limulus* amebocyte lysate were mixed in pyrogen-free test tubes and incubated for 1h at 37°C. The test was judged positive when the gel formed did not collapse upon complete inversion of the tube. Gelation was considered to indicate the presence of endotoxin. In order to quantify the gelation activity of *Limulus* lysate, serial dilutions of each LPS were made in pyrogen-free distilled water and portions of each diluted specimen were assayed as described above. The lowest concentration which caused the gelation of *Limulus* amebocyte lysate was determined. In each assay, pyrogen-free distilled water served as a negative control[10].

Table 1. Comparisons of fatty acid compositions of LPSs from *V. vulnificus*, *E. coli*, and *S. typhimurium*

fatty acid	name	*V. vulnificus* (%)	*E. coli* (%)	*S.typhimurium* (%)
C 8:0	octanoic	7.6	9.6	6.3
C 10:0	capric	24.8	31.8	48.6
C 12:0	lauric	19.8	37.0	25.0
C 14:0	myristic	41.4	6.2	2.6
C 16:0	palmitic	1.1	-	1.8
C 17:0	heptadecanoic	4.4	11.6	9.1
C 18:0	stearic	-	2.9	0.3
C 18:1	oleic	0.9	0.8	-
C 18:2	linoleic	-	-	1.1
C 18:3	linolenic	-	-	5.2

Lethal toxicity

Measurement of the lethal toxicity of LPS was performed with ICR mice. Females aged 7 weeks were used. LPS was dissolved in pyrogen-free phosphate-buffered saline, sonicated for 3 min, and serial dilutions were made in the same saline solution. Groups of 10 mice were injected intravenously with 0.2ml of the serially diluted doses of LPS. Deaths of mouse were recorded daily, the amount of LPS required to kill 50% of the experimental animals(LD_{50}) for each test group was calculated from mortality[11].

Results and Discussion

Fatty acid composition of LPS

The results of fatty acid analyses of LPSs have shown that the major fatty acid for each LPS was myristic acid(C14 : 0) for *V. vulnificus*, lauric acid(C12 : 0) for *E. coli*, and capric acid(C10 : 0) for *S. typhimurium*(Table 1). LPS from *V. vulnificus* did not have the C18-fatty acids (C18 : 0, C18 : 2, and C18 : 3 fatty acid). It was noticeable that the myristic acid was composed as major one(41.4%) for LPS from *V. vulnificus* and that the amount of palmitic acid(C16 : 0) was very little(below 2%) for all LPSs.

Fatty acids were analyzed in a 3mm × 3m glass column packed with 10% 1,4-butanediol succinate on Chromosorb W at 185°C by GC.

Limulus gelation activity

Limulus polyphemus(horseshoe crab) belongs to the arthropod subphylum chelicerata. Its blood(hemolymph) contains only one type of cells, the amebocytes. The lysed amebocytes from *L. polyphemus* were found to form a gel in the presence of minute amounts of endotoxin. *Limulus* gelation test has been used as the most sensitive method for detecting endotoxin in patients with gram-negative septicema and for studying experimental endotoxemia. The constituent of endotoxin that causes the gelation of *Limulus* lysate is a glycophospholipid, designated as lipid A. The result of this test was presented in Table 2. All LPSs from three bacteria were extremely effective in gelating *Limulus* lysate. *Limulus* gelation activity of LPS from *V. vulnificus* was comparable to those of LPS from *E. coli* and *S. typhimurium* (0.1 ng/ml).

Lethal toxicity

LPSs from *V. vulnificus*, *E. coli*, and *S. typhimurium* were tested for their ability to kill mouse when injected intravenously. The lethal toxicity of LPS from *V. vulnificus* was in the same range as those of *E. coli* and *S. typhimurium* which were used as references(Table 2). Neither LPS from *E. coli*, *S. typhimurium* nor *V. vulnificus* was lethal in doses of up to 100 mg/kg mouse. LPS from *V. vulnificus* was less toxic than the other LPSs.

Table 2. Results of *Limulus* lysate test and lethal toxicity of LPS from three bacteria

LPS	*Limulus* activity (ng/ml)[a)]	LD_{50}(mg/kg)
V. vulnificus	0.1[b)]	140
E. coli	0.1	120
S. typhimurium	0.1	100

a) Figures represent the lowest concentration of individual fraction at which gelation of the lysate occurred.
b) Pyrogen-free distilled water was used as the negative control.

It was reported that the weak biological activity of Agrobacterium sp. LPS might be due to its different chemical composition. In contrast to the typical fatty acid pattern of the enterobacterial LPS(3-hydroxymyristic acid accompanied by lauric, myristic, and palmitic acid), the fatty acids of Agrobacterium sp. LPS were consisted solely of the two 3-hydroxy fatty acids(3-hydroxy myristic and palmitic acid) and was devoid of heptose and contained relatively little 2-keto-3-deoxyoctonate(KDO). Therefore, it may be said that the activities of LPS depend on its fatty acid composition.

The variable factors in the biological activity-structure relationship of LPS might be ; (a) the presence or absence of the phosphate groups, (b) the number and kinds of O-acyl fatty acids, and (c) the presence or absence of 4-amino arabinose or pyrophosphorylethanol amine. During the course of studies attempting to correlate biological activity of endotoxin with its structure, attention has been focused on the importance of size or degree of aggregation of the polymer. It was thought that the basic elements of endotoxin were necessarily aggregated into particles of colloidal dimensions before antigenicity and toxic properties could be manifested.

The fatty acid compositions of LPSs from *E. coli, S. typhimurium,* and *V. vulnificus* were analyzed in order to compare the endotoxicity of LPS from *V. vulnificus* with those from *E. coli* and *S. typhimurium.* LPS from *V. vulnificus* had a different fatty acid composition from those of *E. coli* and *S. typhimurium.* The major fatty acid from each LPS was lauric acid for *E. coli,* capric acid for *S. typhimurium,* and myristic acid for *V. vulnificus.* LPS from *V. vulnificus* had been expected to have the strongest activity of three LPSs because the chain of myristic acid was the longest. But the toxicities of LPS from *V. vulnificus* were not the strongest, rather almost the same as those of *E. coli* and *S. typhimurium* in terms of the gelation activity of *Limulus* lysate and lethal toxicity. Therefore, we dare to propose that in addition to LPS, such factor as exotoxin(hemolysin, protease) should be considered as the cause for the cytotoxicity by V. vulnificus.

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초록 : *Vibrio vulnificus* Lipopolysaccharide의 세포 독성 효과와 지방산 조성

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감염성 *Vibrio vulnificus*에 의한 패혈증과 같은 질병의 원인을 알아보기 위하여 *Vibrio vulnificus*로 부터 세포벽 lipopolysaccharide를 추출한 후 지방산 조성을 분석하고 *Limulus* gelation activity와 lethal toxicity를 측정하였다. 이 결과들을 비감염성 *Escherichia coli* LPS와 감염성 *Salmonella typhimurium* LPS의 것들과 비교하였다. *V. vulnificus* LPS의 주 지방산은 myristic acid 이었고 *E. coli* LPS는 lauric acid, *S. typhimurium* LPS는 capric acid 이었다. 세가지 LPS의 *Limulus* gelation activity는 같았으며(0.1ng/ml), *V. vulnificus* LPS의 lethal toxicity는 *E. coli* LPS와 *S. typhimurium* LPS의 것과 비슷하였다. LPS 이외에도 exotoxin과 같은 인자들도 *V. vulnificus*에 의한 세포 독성의 원인으로 고려해야 할 것이다.