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NOTES

Hemagglutination Is a Novel Biological Function of Lipopolysaccharide (LPS), as Seen with the *Vibrio cholerae* O139 LPS

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It has been generally thought that the polysaccharide moiety of lipopolysaccharide (LPS) maintains only serological specificity, while the lipid A portion determines various biological functions. However, we found that hemagglutination was a common function of the polysaccharide moiety of LPSs from important human enteropathogenic bacteria. Of the LPSs examined, *Vibrio cholerae* O139 LPS showed the highest hemagglutinating activity. Glycoproteins, such as mucin and fetuin, showed efficient inhibition of the hemagglutinating ability. Since cell-mediated hemagglutination is known to be correlated with bacterial adherence, hemagglutination induced by the polysaccharide moiety is interpreted to indicate that cell-surface LPS is a potential adhesin.

Human enteropathogens, like *Vibrio cholerae* or *Escherichia coli*, colonize the host intestinal mucosa to cause a diarrheal disease. In the course of a crisis of a diarrheal disease, cell surface components including lipopolysaccharide (LPS) interact directly with the host intestinal mucosa, which results in induction of antibody production (10). The production of antibodies against enterotoxins is evident; however, antibacterial immunity confers more effective protection than the antitoxic immunity (8). During studies with human volunteers, the attenuated live vaccine administered parenterally has protected the population against cholera (12, 13).

The bacterial adherence to the intestinal mucosa is a prerequisite in colonization, and therefore, considerable attention has been given to the factor(s) related to adherence. The adhesive ability of an enteropathogen is usually assessed by determining the hemagglutinating ability, because the erythrocyte membrane is believed to possess the homolog(s) of the mucosal substance(s) involved in bacterial adherence (14, 23, 25). Actually, in many human pathogens, including *Vibrio mimicus*, the hemagglutinating ability of bacterial cells has been shown to be closely correlated with the bacterial ability to adhere to the host intestine (2, 5, 14, 25). We recently isolated two cell-associated hemagglutinins (HAs) from *V. mimicus* E-33 (1), which adheres strongly to the small intestine (2). Additionally, the HAs were found to correspond to a major outer membrane protein and the polysaccharide moiety of LPS (1).

In the present study, we report hemagglutinating ability as a common property of the polysaccharide moiety of LPS isolated from enteric pathogens including *V. cholerae* O139 Bengal, a newly recognized pathogen of the prevailing eighth pandemic of cholera (3, 20). We also report that *V. cholerae* O139 LPS has the highest hemagglutinating activity, suggesting involvement of LPS in the increased pathogenicity of this vibrio.

We (1) recently reported that one of the cell-associated HAs of *V. mimicus* E-33 was LPS and that only the polysaccharide moiety mediated hemagglutination. In order to examine the hemagglutinating ability of other vibrio LPSs, LPS was isolated from several strains including two isolates of *V. cholerae* O139. The vibrio was grown in tryptic soy broth at 37°C overnight with shaking, and the cells were harvested by centrifugation at 6,000 × *g* for 30 min and killed by acetone treatment. The acetone-killed bacterial cells were freeze-dried, and LPS was purified by hot phenol-water extraction followed by repeated ultracentrifugation (24). The purity of each LPS preparation obtained was confirmed by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis followed by silver staining (1). From 2.5 g (dry weight) of the bacterial cells, 5 to 10 mg of LPS was isolated from each vibrio strain.

The hemagglutinating activity of vibrio LPS (500 μg/ml) was assayed by the method of Jones et al. (7). A serial twofold dilution of 50 μl of the sample in 20 mM Tris-HCl buffer containing 0.9% NaCl (pH 7.5) was mixed with 50 μl of 1.5% rabbit erythrocytes in the wells of a 96-well polystyrene V-bottom microtiter plate (Greiner, Nurtingen, Germany). The plate was incubated at room temperature for 45 min, and the hemagglutination was monitored visually. The HA unit was defined as the reciprocal of the highest dilution of the sample causing visible agglutination of the erythrocytes. As shown in Table 1, all vibrio LPS preparations had hemagglutinating activity against rabbit erythrocytes. However, it should be noted that LPS from either of the *V. cholerae* O139 strains showed activity four times greater than others (Table 1).

By using *V. mimicus* E-33 LPS, we previously showed that hemagglutination was elicited by the polysaccharide fraction but not by the lipid A fraction (1). Therefore, in the present study, each of the vibrio LPS preparations was also hydrolyzed with 1% acetic acid at 100°C for 90 min, and the lipid A fraction was spun down (16). Thereafter, the supernatant containing the polysaccharide region was collected and lyophilized, and the hemagglutinating activity was then determined. For *V. mimicus* E-33 LPS, 100% of the hemagglutinating activity was recovered in the polysaccharide fraction (data not

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TABLE 1. Hemagglutinating activities of LPS preparations tested against erythrocytes from different sources[a]

LPS source	Hemagglutinating activity (HAU) with erythrocytes from:	
	Rabbits	Mice
Vibrionaceae		
V. mimicus E-33	5,120	20,480
V. mimicus E-26	2,560	5,120
V. mimicus Pt-48	5,120	20,480
V. cholerae non-O1 ATCC 4716	2,560	5,120
V. cholerae O1 El Tor P-1418	5,120	10,240
V. cholerae O139 MDO 11	20,480	81,920
V. cholerae O139 VO 10	20,480	81,920
Enterobacteriaceae		
Salmonella enteritidis	5,120	10,240
E. coli O128:B12	–[b]	2,560

[a] Each LPS preparation (25 μg) was diluted serially in 20 mM Tris-HCl buffer containing 0.9% NaCl (pH 7.5) and mixed with 1.5% of each erythrocyte suspension. The mixture was incubated at room temperature for 45 min, and the hemagglutination was monitored. The HA unit (HAU) was defined as the reciprocal of the highest dilution of the sample causing visible hemagglutination. Erythrocytes from guinea pigs, sheep, cows, horses, and chickens showed no hemagglutination (i.e., activity of <20 HA units).

[b] Less than 20.

TABLE 2. Inhibitory effects of glycoproteins on hemagglutination induced by vibrio LPS[a]

LPS source	MIC (μg/ml)		
	Mucin	Fetuin	Asialofetuin
V. mimicus E-33	3.1	1.6	1.6
V. mimicus E-26	3.1	1.6	0.8
V. mimicus Pt-48	1.6	12.5	0.8
V. cholerae non-O1 ATCC 4716	1.6	3.1	1.6
V. cholerae O1 El Tor P-1418	1.6	12.5	1.6
V. cholerae O139 MDO 11	3.1	6.3	0.8
V. cholerae O139 VO 10	3.1	6.3	0.8

[a] Each vibrio LPS (1 HA unit) was treated with an appropriate amount of each of the glycoproteins at 37°C for 15 min, and the residual HA activity of the vibrio LPS was then assayed. Thereafter, the MIC of each glycoprotein was estimated.

shown). It is well established that all biological activities of LPS, which include mitogenic, lethal, pyrogenic, and limulus lysate-gelating activities, are mediated exclusively by the lipid A moiety (9). However, the data presented herein clearly demonstrates that hemagglutination is a biological function performed by the polysaccharide moiety.

The HA assay of the LPS preparations from vibrios, as well as from *E. coli* O128:B12 and *Salmonella enteritidis* (Sigma Chemical Co., St. Louis, Mo.), revealed strong hemagglutination of rabbit and mouse erythrocytes, except that *E. coli* LPS agglutinated only mouse erythrocytes (Table 1). However, *E. coli* LPS could also agglutinate rabbit erythrocytes upon hydrolysis with acetic acid and removal of lipid A, indicating that the lipid A moiety may interfere with access of the polysaccharide moiety to the receptor on rabbit erythrocytes. We further studied various LPS preparations from human pathogenic bacteria, such as *V. cholerae* O1 569B, *E. coli* O55:B5 and O26:B6, *Klebsiella pneumoniae*, *Salmonella minnesota*, *Salmonella typhi*, *Shigella flexneri*, and *Serratia marcescens* (Sigma Chemical Co.). It was confirmed that, although LPS from *V. cholerae* O139 Bengal conferred the highest HA activity, hemagglutination was a common biological property of LPS from gram-negative bacteria.

Each vibrio LPS was mixed with an appropriate amount of sugar or glycoprotein and incubated at 37°C for 15 min. Thereafter, the mixture was subjected to the HA assay. Sugars examined were D-arabinose, D-fructose, D-galactose, D-glucose, D-mannose, maltose, lactose, sucrose, *N*-acetylneuraminic acid, *N*-acetylglucosamine, and *N*-acetylgalactosamine. Also, glycoproteins used were mucin (bovine submaxillary), fetuin (fetal calf serum), and asialofetuin (fetal calf serum). Analogous to the results obtained in our recent study using *V. mimicus* E-33 LPS (1), hemagglutination induced by any vibrio LPS was also inhibited by the glycoprotein in a dose-dependent manner, while no sugars showed significant inhibitory effects even when as high a concentration as 10 mg/ml was added to the assay system (data not shown). The glycoprotein was serially diluted and incubated with each vibrio LPS at 37°C for 15 min, and the residual HA activity of vibrio LPS against rabbit erythrocytes was then determined. Thereafter, the MIC of each of the glycoproteins was estimated. As shown in Table 2, mucin and asialofetuin efficiently abolished the HA activity of all of the vibrio LPS preparations, but some LPSs were found to be considerably resistant to fetuin.

In order to test the LPS-inactivating potential of the polypeptide portion of the glycoprotein, exhaustive pronase digestion of the glycoprotein was carried out as described by Saha and Banerjee (17). Mucin, fetuin, or asialofetuin was mixed with pronase at a ratio of 50:1 in 50 mM Tris-HCl buffer supplemented with 1 mM $CaCl_2$ (pH 8.0), and the mixture was incubated at 37°C for 72 h. Thereafter, pronase was inactivated by heating at 80°C for 20 min, and each of the digested glycoproteins was subjected to the LPS-inactivating assay. It was shown that no pronase-digested glycoprotein had the LPS-inactivating ability. The glycoproteins were also digested with a glycolytic enzyme, such as neuraminidase, β-D-galactosidase, or β-D-glucosidase. In contrast to the pronase digestion, the digestion with the glycolytic enzyme caused no reduction of the LPS-inactivating potential of any glycoprotein. These results obviously indicate involvement of the polypeptide portion of the glycoprotein in interaction with vibrio LPS. The diverse group of bacterial HAs known so far are proteinous substances binding specifically to carbohydrates (15, 17), and so vibrio LPS, a nonproteinous HA, appeared to be unique in its binding to polypeptides. The inability of any sugar to abolish the adherence of *V. cholerae* O1 to the mucous coat of the human small intestine was documented by Yamamoto and Yokota (26). This suggests involvement of the nonsugar mucosal component(s) in the adherence of this human pathogen. Since the major part of the small intestine is covered with a thick mucous layer containing glycoproteins (23, 25), sufficient elimination of the hemagglutinating ability of any vibrio LPS with the polypeptide portion of the glycoprotein may indicate the possibility of LPS-mediated intestinal adherence in the enteropathogenic vibrios, including *V. cholerae* O1.

V. cholerae O139 Bengal endangers human lives and is currently the subject of extensive global studies because of its extraordinary ability to cause cholera. Studies of its pathogenicity suggest a resemblance between O139 Bengal and O1 biotype El Tor (3, 20). The most notable finding in the present study may be the high HA activity of *V. cholerae* O139 LPS. Oral immunization against *V. cholerae* O139 has implied that LPS conferred the most efficient protection (3, 4). However, a study on biological activities of O139 LPS suggested no contribution of the lipid A moiety in the high pathogenic potential of this vibrio (21). It is therefore plausible that, as cholera researchers worldwide search clues to what makes O139 Ben-

gal so efficient in its pathogenicity, the high hemagglutinating ability of the polysaccharide moiety may be of interest.

The correlation between cell-mediated hemagglutination and intestinal adherence in several enteropathogenic vibrios has been established (2, 14, 23, 25). *V. cholerae* has been reported to express rigid pili controlled by a transmembrane protein, ToxR, which also controls expression of cholera toxin (11). This toxin-coregulated pilus is known to have a role in bacterial adherence (6), but this pilus is restricted to *V. cholerae* serogroups, O1 (6) and O139 (19). The hemagglutinating function of outer membrane proteins has also been documented (1, 18, 22). However, considering the cell surface architecture, direct interaction of the outer membrane protein with the host mucosa seems to be impossible. In contrast, the extended polysaccharide moiety of LPS may be the first to come into contact with the mucosal membrane. On balance, it seems likely that, although the toxin-coregulated pili may be essential for the cholera vibrios, the polysaccharide moiety with the strong hemagglutinating ability may function as the ubiquitous adhesin in the enteropathogenic vibrios.

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