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Vibrio mimicus Attaches to the Intestinal Mucosa by Outer Membrane Hemagglutinins Specific to Polypeptide Moieties of Glycoproteins

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***Vibrio mimicus* is the closest organism to *Vibrio cholerae*. *V. mimicus* E-33, which is a highly adhesive and enteropathogenic strain, is known to produce three types of hemagglutinins (HAs), i.e., a 31-kDa exocellular metalloprotease (Vm-HA/protease), lipopolysaccharide (Vm-LPSHA), and a 39-kDa major outer membrane protein (Vm-OMPHA). Hemagglutination induced by Vm-LPSHA and Vm-OMPHA was inhibited by glycoproteins, including mucin, fetuin, and asialofetuin, but not by monosaccharides, disaccharides, or *N*-acetylated saccharides. The inhibitory potential of each glycoprotein for Vm-OMPHA was greatly augmented by treatment with a glycolytic enzyme such as β-D-galactosidase or β-D-glucosidase, while pronase treatment achieved complete abolition of the inhibitory potential. The inhibitory ability of the glycoproteins for Vm-LPSHA was also abolished by pronase treatment; however, glycolytic enzyme treatment showed no effect. Hence, the polypeptide portion of glycoproteins may directly associate with Vm-OMPHA and Vm-LPSHA, but the sugar moiety may act as a barrier to interaction with Vm-OMPHA. The glycoproteins as well as Fab antibodies against Vm-OMPHA and Vm-LPSHA eliminated the ability of E-33 cells to agglutinate rabbit erythrocytes and to attach to rabbit intestinal mucosa. Additionally, expression of the hemagglutinating ability by the bacterial cells was accompanied by efficient bacterial adherence to the intestinal mucosa. Finally, the hemagglutinating activity of Vm-OMPHA was markedly increased by incubation with Vm-HA/protease. These results indicate that all three HAs may have significant roles in the glycoprotein-mediated intestinal adherence of *V. mimicus* E-33.**

Vibrio mimicus, which had been considered a non-sucrose-fermenting group of *Vibrio cholerae*, has recently emerged as a human gastrointestinal pathogen of the genus *Vibrio* (5, 20). The clinical symptom of the illness is watery to dysentery-like diarrhea, suggesting production of many kinds of virulence factors (5, 20). The following exocellular factors have actually been isolated from this species: enterotoxins closely related to cholera toxin (9, 21) and *Escherichia coli* heat-stable enterotoxin (4), heat-stable (13) and heat-labile (17a) enterotoxic hemolysins causing fluid accumulation, and a metalloprotease (Vm-HA/protease) having hemagglutinating activity (6). Furthermore, *V. mimicus* is known to elicit cell-mediated hemagglutination (24) and attachment to rabbit small intestine (22). Although adherence to the intestinal mucosa is a crucial step in a diarrheal disease, the principle(s) involved in the intestinal adherence of and the cell-mediated hemagglutination by *V. mimicus* remains unknown.

The bacterial hemagglutinin (HA) causing agglutination of erythrocytes has been proposed to be active in bacterial adherence to the intestinal mucosa, because the erythrocyte membrane has been believed to possess the homolog(s) of the mucosal substance(s) involved in adherence (11, 18, 25). Our study on expression of the virulence-related potential of many *V. mimicus* strains revealed a significant correlation between cell-mediated hemagglutination and bacterial adherence to the intestinal mucosa (2). Alam et al. (1) recently isolated two non-protease HAs from the stationary-phase culture supernatant of *V. mimicus* E-33. This strain is negative for production of enterotoxins and the heat-stable hemolysin; however, it shows sufficient enteropathogenicity because of the production of the heat-labile hemolysin (7, 17a). Additionally, these two isolated HAs were demonstrated to be originally cell surface components, namely, lipopolysaccharide (LPS) and a 39-kDa major outer membrane protein (1), so that we herein designate them Vm-LPSHA and Vm-OMPHA, respectively.

In the present communication, we provide additional data on properties of these two *V. mimicus* HAs as well as Vm-HA/protease, which was also isolated from strain E-33, focusing particularly on their roles in bacterial adherence to the intestinal mucosa.

MATERIALS AND METHODS

Chemicals. β-D-Galactosidase and β-D-glucosidase were purchased from Wako Pure Chemicals (Osaka, Japan). Asialofetuin (type I; fetal calf serum), fetuin (fetal calf serum), mucin (type IS; bovine submaxillary glands), neuraminidase, and pronase were obtained from Sigma Chemical (St. Louis, Mo.).

***V. mimicus* HAs.** Vm-HA/protease was purified by the procedure of Chowdhury et al. (6). The 24-h culture supernatant from strain E-33 was fractionated with ammonium sulfate and chromatographed successively on a Sephacryl S-100 and a Mono Q HR 5/5 column in a fast-performance liquid chromatography system (Pharmacia Biotechnology, Uppsala, Sweden). Vm-LPSHA and Vm-OMPHA were purified by a procedure described recently (1). Briefly, the 32-h culture supernatant was concentrated with a Diaflo YM 30 membrane (Amicon, Beverly, Mass.) and treated with 1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate}. The CHAPS-treated sample was chromatographed on a Sephacryl S-400 HiLoad 26/60 column (Pharmacia Biotechnology) and a Fractogel EMD TMAE 650 column (Merck, Darmstadt, Germany) in the fast-performance liquid chromatography system.

The Fab antibodies against Vm-LPSHA and Vm-OMPHA. An adult rabbit was injected subcutaneously with 100 μg of the HA emulsified in an equal volume of Freund's complete adjuvant. After three immunizations, the antiserum was collected. The immunoglobulin M antibody against Vm-LPSHA was purified by ammonium sulfate precipitation followed by dialysis against distilled water. Thereafter, the Fab antibody was prepared by pepsin digestion and column chromatography on a Sephacryl S-200 column. The immunoglobulin G antibody against Vm-OMPHA was obtained by ammonium sulfate fractionation and col-

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umn chromatography on a HiTrap protein A column (Pharmacia Biotechnology). The Fab antibody was then prepared by papain digestion and column chromatography on a HiTrap protein A column. These Fab antibodies were stored at −70°C until used.

Digestion of glycoproteins. Each of the glycoproteins (mucin, fetuin, and asialofetuin) was subjected to digestion with a glycolytic enzyme such as neuraminidase, β-D-galactosidase, or β-D-glucosidase at a ratio (substrate to enzyme) of 25:1 in 20 mM Tris-HCl buffer (pH 8.0) for 30 min at 37°C. After incubation, the glycolytic enzyme was destroyed by heating at 75°C for 30 min, and the sugars liberated from the glycoprotein were removed by dialysis against the buffer.

Exhaustive pronase digestion was carried out by the procedure described by Saha and Banerjee (19). The glycoprotein was mixed with pronase at a ratio of 50:1 in 50 mM Tris-HCl–1 mM $CaCl_2$ buffer (pH 8.0), and the mixture was incubated at 37°C for 72 h. After digestion, pronase was inactivated by heating at 80°C for 20 min.

Assay for hemagglutination. The assay technique was adapted from the method of Jones et al. (15). A serial dilution of 50 μl of the HA in KRT buffer (128 mM NaCl, 5.1 mM KCl, 1.34 mM $MgSO_4$, 2.7 mM $CaCl_2$, 10 mM Tris-HCl [pH 7.5]) was mixed with 50 μl of 1.5% rabbit erythrocytes in the well of a 96-well V-bottom microtiter plate (Greiner, Nurtingen, Germany). The mixture was incubated at 25°C for 45 min, and agglutination of the erythrocytes was monitored visually. The HA unit (HAU) was defined as the reciprocal of the highest dilution of the sample causing visible agglutination of the erythrocytes.

For the determination of the MIC of the glycoproteins, each of the three glycoproteins (mucin, fetuin, and asiolofetuin) was serially twofold diluted and allowed to act on Vm-LPSHA (1 HAU) or Vm-OMPHA (1 HAU) at 37°C for 15 min. Thereafter, the residual hemagglutinating activity of the HA was measured, and the MIC of each glycoprotein was estimated.

The hemagglutinating ability of E-33 cells was tested as follows. *V. mimicus* E-33 was cultivated in tryptic soy broth at 37°C. At 3, 12, or 24 h of cultivation, the bacterial cells were harvested by centrifugation. The cells were washed twice with KRT buffer and resuspended (10^9 cells/ml) in the buffer, and the hemagglutinating activity was assayed with 1.5% erythrocytes in a V-bottom microtiter plate.

Measurement of adherence to the intestinal mucosa (2). An adult rabbit was sacrificed by intravenous injection of pentobarbital sodium (Nembutal; Abbott). The small intestine was excised, opened by longitudinal incision, washed with saline, and fixed overnight with 10% formalin. This formalin-fixed intestine was washed with KRT buffer and punched into equal pieces (7-mm circles). Thereafter, the intestinal piece prepared was dipped in the suspension of E-33 cells (10^9 cells in 1 ml of KRT buffer) and incubated at 30°C for 15 min with mild agitation. After incubation, the intestinal piece was taken out, washed with KRT buffer to remove the nonadherent vibrios, and homogenized in a tissue homogenizer (Nihonseiki Kaisha Ltd., Tokyo, Japan). The number of adherent vibrios (adherence index) was then determined by culturing on nutrient agar plates.

RESULTS

Hemagglutination and intestinal adherence by *V. mimicus* whole cells. Our recent study using many *V. mimicus* isolates demonstrated a significant correlation between the ability to induce cell-mediated hemagglutination and the ability to adhere to the small intestine (2). This finding was further supported by the present study. Whole cells of *V. mimicus* E-33 grown for 3 h showed high cell-mediated hemagglutination toward rabbit erythrocytes and effective adherence to rabbit intestinal mucosa, while the cells grown for 24 h caused poor hemagglutination and adherence. Namely, the bacterial cells (10^9 cells) from 3 h of cultivation had an activity of 32 HAU and an adherence index of 3.5×10^6, while bacterial cells from 24 h of cultivation had an activity of 8 HAU and an adherence index of 2.0×10^6. The maximal adherence of and hemagglutination by E-33 cells grown for 3 h was observed at pH 8.0 (data not shown).

Hemagglutination induced by *V. mimicus* E-33 cells was inhibited only by a glycoprotein like mucin, fetuin, or asialofetuin and not by any simple sugars (data not shown). This bacterium is known to produce two glycoprotein-sensitive HAs, i.e., Vm-LPSHA and Vm-OMPHA, on the outer membrane (1). Thus, these HAs are recognized as being essential not only to cell-mediated hemagglutination but also to adherence to the intestinal mucosa. To confirm this possibility, we examined inhibitory effects of the glycoproteins on the intestinal adherence of E-33 cells from 3 h of cultivation. As shown in Table 1, all glycoproteins significantly reduced the adherence of the bacterial cells to isolated rabbit intestinal mucosa. The bacterial adherence was also significantly inhibited by the Fab antibody against either of the HAs in a dose-dependent manner (Fig. 1). On balance, it may be concluded that both Vm-LPSHA and Vm-OMPHA expressed on the bacterial cell surface are involved in the adherence of *V. mimicus* E-33 to the intestinal mucosa.

TABLE 1. Inhibition of adherence of *V. mimicus* E-33 to the intestinal mucosa by glycoproteins[a]

Glycoprotein (concn)	Adherence (10^5 cells)	*P*
None	159 ± 13	
Mucin (31 μg/ml)	99 ± 21	<0.05
Fetuin (16 μg/ml)	83 ± 21	<0.01
Asialofetuin (16 μg/ml)	110 ± 16	<0.05

[a] The intestinal piece was dipped into the suspension of bacterial cells obtained at 3 h of cultivation. After incubation at 30°C for 15 min, the number of adherent vibrios was determined. The mean ± standard deviation of three separate experiments is presented.

Sugar-independent hemagglutination by Vm-LPSHA and Vm-OMPHA. We reported that hemagglutination by Vm-LPSHA and Vm-OMPHA was not inhibited by a monosaccharide like glucose, galactose, or mannose (1). Other saccharides were also tested for their inhibitory effects on the hemagglutinating activity of either of the HAs. Again, neither Vm-LPSHA nor Vm-OMPHA was inactivated by treatment with a simple sugar (data not shown). The sugars used were D-arabinose, D-fructose, *N*-acetyl neuraminic acid, maltose, lactose, sucrose, *N*-acetylglucosamine, and *N*-acetylgalactosamine. Such inability of a number of simple saccharides allowed us to speculate that both Vm-LPSHA and Vm-OMPHA may recognize the protein portion of the glycoprotein, and the following experiments were designed to clarify this. Each glycoprotein was subjected to digestion with a glycolytic enzyme, and the ability of the digested products to inhibit hemagglutination by Vm-LPSHA and Vm-OMPHA was then determined. As apparent from the

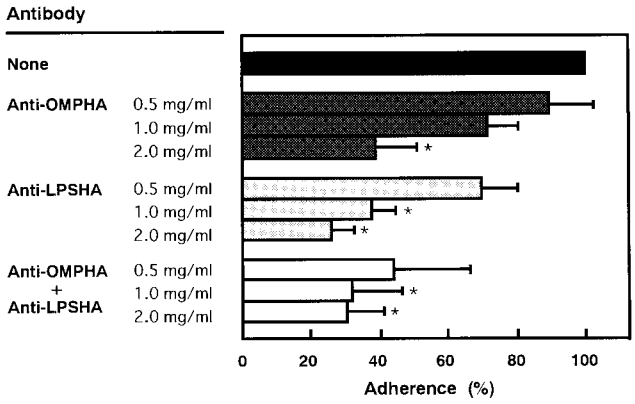

FIG. 1. Inhibition of adherence of E-33 cells to rabbit intestinal mucosa with Fab antibody against Vm-OMPHA or Vm-LPSHA. The bacterial cells (10^9 cells) grown for 3 h were suspended into 1 ml of KRT buffer containing either or both of the Fab antibodies. The intestinal piece was dipped into the bacterial suspensions and incubated at 30°C for 15 min with mild agitation. After incubation, the intestinal piece was taken out, washed with KRT buffer, and homogenized, and the number of adherent bacteria was determined. The number of bacteria adhering to the intestinal mucosa in the absence of the antibody was 159 ± 13 (10^5 cells). The mean values and standard deviations of three separate experiments are shown. *, $P < 0.01$ versus the bacterial number adhering in the absence of the antibody.

TABLE 2. Inhibitory potentials of glycosidase-digested glycoproteins on hemagglutination induced by Vm-OMPHA or Vm-LPSHA[a]

Glycoprotein	MIC (μg/ml)	
	Vm-LPSHA	Vm-OMPHA
Nondigested		
Mucin	3.1	1.6
Fetuin	1.6	0.78
Asialofetuin	1.6	0.10
Neuraminidase digested		
Mucin	3.1	1.6
Fetuin	1.6	0.78
Asialofetuin	1.6	0.10
β-Glucosidase digested		
Mucin	6.3	0.10
Fetuin	1.6	0.012
Asialofetuin	1.6	0.0015
β-Galactosidase digested		
Mucin	3.1	0.20
Fetuin	1.6	0.049
Asialofetuin	3.1	0.0031

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

TABLE 3. Hemagglutinating activities of Vm-HA/protease and native and protease-treated Vm-OMPHAs

Type of erythrocyte	Hemagglutinating activity (HAU)		
	Vm-HA/protease[a]	Native Vm-OMPHA[b]	Protease-treated Vm-OMPHA[c]
Rabbit	—[d]	640	5,120
Chicken	40	160	5,120
Mouse	—	40	160
Guinea pig	—	20	160
Bovine	—	—	—
Horse	—	—	—
Sheep	—	—	—

[a] Purified Vm-HA/protease (1.0 μg) was allowed to act on each of the erythrocytes.
[b] Purified Vm-OMPHA (3.0 μg) was allowed to act on each of the erythrocytes.
[c] The Vm-HA/protease (1.0 μg) and Vm-OMPHA (3.0 μg) were mixed and incubated at 37°C for 15 min. After incubation, the HA/protease was completely inactivated by heat treatment, and the hemagglutinating activity was measured.
[d] —, less than 10 HAU.

results shown in Table 2, digestion of the sugar portion did not affect the inhibitory potential of any glycoprotein for Vm-LPSHA, implying that the sugar portion may not be involved in the interaction with Vm-LPSHA. On the other hand, digestion with β-D-glucosidase or β-D-galactosidase, but not with neuraminidase, elicited a significant increase in the inhibitory potential for Vm-OMPHA, suggesting that the sugar moiety interfered with the association of the glycoprotein with Vm-OMPHA. Conversely, when the protein portion was exhaustively digested by pronase, all glycoproteins failed to inhibit the hemagglutinating action of the HAs (data not shown), which implied a critical involvement of the polypeptide moiety in interaction with Vm-OMPHA and Vm-LPSHA.

Direct activation of Vm-OMPHA by Vm-HA/protease. The Vm-HA/protease causing hemagglutination of chicken erythrocytes was recently found to stimulate the intestinal adherence of *V. mimicus* E-33 (3). Therefore, we speculated that Vm-HA/protease may directly interact with and activate Vm-OMPHA. The Vm-HA/protease (1 μg) was mixed with Vm-OMPHA (3 μg), and the mixture was allowed to incubate at 37°C for 30 min. After incubation, the HA/protease was completely inactivated by heating at 65°C for 30 min, and the hemagglutinating activity of the HA/protease-treated Vm-OMPHA was measured with the appropriate erythrocytes. As shown in Table 3, the hemagglutinating ability for several kinds of erythrocytes, which are sensitive to native Vm-OMPHA, was augmented significantly by treatment with Vm-HA/protease. However, such activation was not observed in the presence of the antibody against the HA/protease or a metalloprotease inhibitor like EGTA or phosphoramidon, indicating involvement of the proteolytic action in activation of the OMPHA (data not shown). In contrast, bovine, horse, and sheep erythrocytes were not agglutinated, even when the HA/protease-treated Vm-OMPHA was allowed to act on the erythrocytes, suggesting that the HA/protease treatment did not affect the receptor specificity of Vm-OMPHA. The Vm-HA/protease activated the OMPHA in a dose- and time-dependent manner; however, a significant change in the molecular mass of Vm-OMPHA was not observed when it was analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol (data not shown). The study to clarify the activation mechanism is in progress.

DISCUSSION

In a number of human intestinal pathogens including *V. mimicus*, the cell-associated HA(s) has been documented as a possible factor in bacterial adherence to the intestinal mucosa (2, 10, 18, 24, 25). With *V. cholerae*, extensive studies on cell-mediated hemagglutination have demonstrated the involvement of pili or fimbriae in hemagglutination (16). Although the bacterial cells showed strong hemagglutination and efficient intestinal adherence, *V. mimicus* E-33 expressed no pili on the cell surface (1). Thus, it is interpreted that this strain employs a nonfimbrial substance(s) as the cell surface adhesin(s). We isolated two nonfimbrial outer membrane HAs, Vm-LPSHA and Vm-OMPHA, and found that both were inactivated by glycoproteins like mucin, fetuin, and asialofetuin (1). The present study showed that these glycoproteins, as well as Fab antibodies against Vm-LPSHA and Vm-OMPHA, significantly inhibited cell-mediated hemagglutination and bacterial adherence to the intestinal mucosa. Hence, both Vm-LPSHA and Vm-OMPHA inevitably appear to contribute to hemagglutination by and adherence of *V. mimicus* E-33 cells. Although induction of an immune response by outer membrane components has been well documented (8, 17), the recognition of mediation of hemagglutination and bacterial adherence may provide a new basis for understanding the biological functions of outer membrane components.

The hemagglutinating actions of Vm-LPSHA and Vm-OMPHA were inhibited by glycoproteins even if they were digested with a glycolytic enzyme like neuraminidase, β-D-galactosidase, or β-D-glucosidase. This indicates that the saccharide moiety of the glycoprotein does not promote interaction with either of the HAs. Consequently, the failure of the pronase-digested glycoprotein to inhibit the hemagglutinating action is suggestive of an acute involvement of the polypeptide moiety in binding to Vm-LPSHA and Vm-OMPHA. On the other hand, the increase in the inhibitory potential of the glycolytic enzyme-treated glycoprotein for Vm-OMPHA sug-

gests that the branched sugar moiety may serve as a barrier to access to the glycoprotein by the OMPHA. Diverse groups of bacterial HAs bind to carbohydrates (10, 11, 18). Therefore, the data presented herein is the first description of a proteinaceous substance in the binding site on the erythrocyte membrane for bacterial HAs.

The LPS-binding protein in the human body is a glycoprotein with the ability to potentiate the bactericidal activity of human bactericidal or permeability-increasing protein through specific binding to LPS (14, 23). Although it is not known whether the homolog(s) of the LPS-binding protein is contained in the mucosa of human small intestine, the hemagglutinability and the affinity to glycoproteins may indicate that gram-negative bacterial enteropathogens including *V. mimicus* employ LPS in the glycoprotein-mediated anchorage to the host intestinal mucosa.

Toxin-coregulated pili may be essential for the intestinal colonization of cholera vibrio (12). However, the recent related knowledge advocates that adherence of cholera vibrio to the intestinal mucosa is multifactorial (16). The present study clearly indicated significant roles of two outer membrane HAs, Vm-LPSHA and Vm-OMPHA, in adherence of *V. mimicus* E-33. In addition, activation of Vm-OMPHA by Vm-HA/protease indicates the possible involvement of HA/protease in bacterial adherence. Taken together, these results are interpreted to firmly indicate that adherence of *V. mimicus* E-33 is also multifactorial.

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