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Purification and Characterization of a Hemolysin Produced by *Vibrio mimicus*

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Vibrio mimicus **is a causative agent of human gastroenteritis. This pathogen secretes a pore-forming toxin, *V. mimicus* hemolysin (VMH), which causes hemolysis by three sequential steps: binding to an erythrocyte membrane, formation of a transmembrane pore, and disruption of the cell membrane. VMH with a molecular mass of 63 kDa was purified by ammonium sulfate precipitation and column chromatography with phenyl Sepharose HP and Superose 6 HR. The hemolytic reaction induced by VMH continued up to disruption of all erythrocytes in the assay system. Moreover, VMH that bound preliminarily to erythrocyte ghosts showed a sufficient ability to attack intact erythrocytes. These results suggest reversible binding of the toxin molecule to the membrane. The final cell-disrupting stage was effectively inhibited by various divalent cations. Additionally, some cations, such as Zn^{2+} and Cu^{2+}, blocked the pore-forming stage at high concentrations. Although VMH could disrupt all kinds of mammalian erythrocytes tested, those from horses were most sensitive to the hemolysin. Horse erythrocytes were found to have the most toxin-binding sites and to be hemolyzed by the least amount of membrane-bound toxin molecules, suggesting that toxin binding to and pore formation on erythrocytes are more effective in horses than in other mammals. Purified VMH induced fluid accumulation in a ligated rabbit ileal loop in a dose-dependent manner. In addition, the antibody against the hemolysin obviously reduced enteropathogenicity of living *V. mimicus* cells. These findings clearly demonstrate that VMH is probably involved in the virulence of this human pathogen.**

Vibrio mimicus, a species closely related to *Vibrio cholerae* (8), is a causative agent of human gastroenteritis. The clinical symptom of the illness is watery to dysentery-like diarrhea (13), suggesting that this pathogen produces many kinds of virulence factors. Enterotoxins similar to cholera toxin (4, 6, 35) and heat-stable enterotoxin (11, 27) have been isolated from some clinical strains as causative factors of watery diarrhea. However, most virulent strains lack the ability to produce any enterotoxins. With *Vibrio parahaemolyticus*, the good correlation between enteropathogenicity and hemolysin production is well known (18, 28). On the other hand, for *V. cholerae*, the production of an enterotoxic hemolysin in addition to cholera toxin has been documented (15). Previously, Honda et al. (14) reported production of two types of hemolysin by *V. mimicus*. One hemolysin was heat labile and immunologically similar to *V. cholerae* hemolysin, while another was heat stable and closely related to the thermostable direct hemolysin produced by *V. parahaemolyticus*. These findings suggest that the hemolysins produced by *V. mimicus* are also virulence determinants, especially in dysentery-like diarrhea. The heat-stable *V. mimicus* hemolysin was previously purified and characterized by Yoshida et al. (44), while the purification of the heat-labile hemolysin has not yet been achieved.

Shinoda et al. (33) studied the hemolytic mechanism of the heat-labile *V. mimicus* hemolysin (VMH). VMH was indicated to be a member of pore-forming hemolysins, because the toxin formed a transmembrane pore with a diameter of ca. 3 nm. The pore formed by VMH was permeable to water and monovalent ions, such as Na^+ and K^+, but impermeable to hemoglobin. This finding suggests that VMH causes hemolysis in a colloid osmotic manner. In this hemolytic manner (1), extracellular water comes into the permeated erythrocyte via the pore, which elicits the physical explosion of the erythrocyte membrane from increased intracellular colloidal osmotic pressure due to pore-impermeable hemoglobin.

In this paper, we describe the purification procedure for VMH, the hemolytic reaction induced by the toxin, and the enterotoxic ability of purified VMH.

MATERIALS AND METHODS

Bacterial strain and cultivation medium. *V. mimicus* E-33, an environmental isolate, was used in our experiments. This strain shows enteropathogenicity in the rabbit ileal loop test, in spite of its inability to produce either enterotoxins or the heat-stable hemolysin (3).

The dialysate of heart infusion broth supplemented with 6.6 mM EGTA and 1% NaCl was used for cultivation of the bacterium. EGTA, an inactivator of *V. mimicus* metalloprotease (5), was added to the medium to exclude the possibility of the exocellular proteolysis of the hemolysin (12).

Purification of VMH. The bacterium was cultivated at 37°C for 24 h with shaking (140 cycles/min), and the culture supernatant was collected by centrifugation at 7,000 × *g* for 40 min. After addition of 0.04% NaN_3, the supernatant was concentrated by ultrafiltration with a Diaflo membrane of type YM 30 (Amicon, Beverly, Mass.). Thereafter, ammonium sulfate was added to 70% saturation (472 g/liter). The resulting precipitate was collected by centrifugation at 7,000 × *g* for 40 min, washed once with 70%-saturated ammonium sulfate, and dissolved in 10 ml of 10 mM Tris-HCl buffer (pH 7.5).

The crude VMH preparation thus obtained was applied to a HiLoad 16/10 phenyl Sepharose HP column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 10 mM Tris-HCl buffer (pH 7.5). Bulk protein which did not associate with the gel was washed out with 150 ml of the same buffer, and then VMH was eluted with 20 mM Tris-HCl buffer (pH 8.5). Fractions containing VMH were collected and concentrated to less than 0.5 ml on a YM 30 membrane. Thereafter, the concentrated preparation was mixed with 1.5 ml of 10 mM glycine buffer containing 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS, pH 10; Wako Pure Chemical, Osaka, Japan), and the mixture was incubated at 4°C overnight.

The CHAPS-treated preparation was loaded on a Superose 6 HR 10/30 column (Pharmacia Biotech) equilibrated with 10 mM glycine buffer containing 1% CHAPS and 20% ethylene glycol (pH 10.0). VMH was eluted with the same buffer at a flow rate of 0.5 ml/min. The fractions containing VMH were collected, diluted 10-fold with glycine buffer (pH 10.0), and ultrafiltrated on a YM 30

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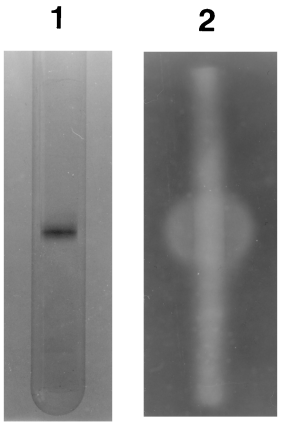

FIG. 1. CHAPS-PAGE of VMH. The VMH preparation (8 μg) obtained by Superose 6 column chromatography was applied to a 6% polyacrylamide gel containing 0.5% CHAPS. In lane 1, the gel was stained with 0.5% Coomassie brilliant blue. In lane 2, the gel was embedded in the agar plate containing 1% horse erythrocytes and the plate was incubated at room temperature. The hemolytic zone was observed in the area corresponding to the protein band.

membrane. To reduce the concentration of CHAPS to less than 0.005%, which has no effect on the hemolytic reaction, washing with glycine buffer (pH 10.0) was repeated once more. The final VMH preparation thus obtained was stored at −20°C until use.

Preparation of antibodies. Immunoglobulin G (IgG) antibody against VMH was prepared as follows: 0.4 mg of the purified hemolysin was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into the back skin of a rabbit (2.5 kg) at 2-week intervals. The antiserum was obtained 7 days after the third injection, and the IgG fraction was purified by ammonium sulfate fractionation. This IgG preparation was used as IgG antibody against VMH. Control IgG was prepared by immunization with the only adjuvant as described previously (25). The protein contents of both IgG preparations were adjusted to 50 mg/ml with saline, and 0.5 ml of the IgG antibody was found to be able to neutralize 0.1 mg of purified VMH. The specificity of the IgG antibody was confirmed by immunoblotting with purified and crude VMH preparations, in which the antibody reacted with a single protein.

Preparation of erythrocyte ghosts. Horse erythrocyte ghosts were prepared by the method of Tomoda et al. (37). The washed erythrocytes were disrupted at 0°C by shaking with a 20-fold volume of 5 mM Tris-HCl buffer (pH 8.0) for 5 min. Thereafter, a 1/10 volume of 1 M glucose was added to the preparation of the disrupted cells (ghost cells) and the ghost cells were isolated by centrifugation (12,000 × *g* for 5 min) followed by washing with 5 mM Tris-HCl buffer (pH 8.0).

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (17) with 10% acrylamide gel. After electrophoresis, the gel was stained with 0.5% Coomassie brilliant blue. CHAPS-PAGE was carried out essentially according to the method of Davis and Ornstein (7), but 6% acrylamide gels were supplemented with 0.5% CHAPS. After electrophoresis, one gel was stained with 0.5% Coomassie brilliant blue and another gel was embedded into the agar plate containing 1% horse erythrocytes. The blood agar plate was incubated at room temperature for an appropriate period.

Determination of protein amount. Protein amounts were determined by the method of Lowry et al. (21), and bovine serum albumin (type I; Sigma Chemical Co., St. Louis, Mo.) was used as a standard protein.

Assay of the hemolytic activity. Unless otherwise stated, hemolytic activity was determined with 1% horse erythrocytes as described previously (32). Briefly, the hemolysin was diluted serially with 10 mM Tris-HCl buffer containing 0.9% NaCl and 0.1% bovine serum albumin (fraction V, pH 7.5; Sigma Chemical). Each of the samples (1 ml) was mixed with horse erythrocytes (1 ml) and incubated at 37°C for 1 h. Thereafter, the reaction mixtures were centrifuged at 1,000 × *g* for 5 min and the amount of hemoglobin released from disrupted erythrocytes was determined by measuring the absorbance at 540 nm of the supernatants. One hemolysin unit (HU) was defined as the amount of hemolysin eliciting 50% hemoglobin release.

Assay of enterotoxic activities with rabbit ileal loops. For the study of enterotoxicity of the purified toxin, an appropriate amount of VMH (1 ml) in phosphate-buffered saline containing 1% gelatin (pH 7.5) was injected into a ligated rabbit ileal loop as previously described (15). At 8 h postinjection, the animal was sacrificed and the extent of fluid accumulation was observed.

The ileal loop tests with *V. mimicus* cells were carried out as follows: living or Formalin-killed cells (10^8 cells in 0.5 ml of fivefold-diluted heart infusion broth) were mixed with 0.5 ml of the IgG antibody, control IgG, or saline. Each of the samples was inoculated into a ligated ileal loop, and the extent of fluid accumulation was observed at 18 h postinjection.

In each experiment, the fluid-accumulating activity was expressed as the ratio of fluid volume (in milliliters) per loop length (in centimeters).

RESULTS

Purification of VMH. Crude VMH was applied to a phenyl Sepharose column equilibrated with 10 mM Tris-HCl buffer at pH 7.5. At this pH, most materials other than VMH did not associate with the gel. Then, associated VMH was eluted with 20 mM Tris-HCl buffer (pH 8.5). The fractions containing VMH were collected and treated with 1% CHAPS. This CHAPS-treated preparation was loaded on a Superose 6 column equilibrated with 10 mM glycine buffer containing 1% CHAPS and 20% ethylene glycol (pH 10.0). The elution profile showed that only the major 63-kDa protein peak had hemolytic activity.

As described, gel filtration was carried out in the presence of CHAPS, because the VMH molecule was found to interact with Superose 6 gel. The interaction of *V. cholerae* hemolysin with Sephadex G-100 gel is able to be impeded with 0.25 M glucose (40). However, this saccharide failed to overcome the affinity of the VMH molecule with the Superose gel. Among various detergents tested, only CHAPS blocked the interaction without loss of hemolytic activity.

CHAPS-PAGE of the final preparation showed a single protein band corresponding to the hemolytic activity (Fig. 1). SDS-PAGE with or without 2-mercaptoethanol also gave a sole protein band with a molecular mass of 63 kDa, indicating that VMH is a single-chain polypeptide (data not shown). Since the molecular weights of VMH determined by SDS-PAGE and gel filtration were comparable, it is evident that VMH is a monomeric toxin. Thus, the final VMH preparation was considered to be highly homogeneous. Consequently, this toxin preparation was used as a purified VMH preparation in the following experiments.

The amounts of protein recovered and the hemolytic activities are shown in Table 1. From 2.4 liters of the culture supernatant, 0.3 mg of purified VMH was obtained, and the percentage of recovery of the activity was 7.2%. The specific activity was increased about 2,800 times.

Kinetics of hemolytic reaction. Shinoda et al. (33) reported that lysis of horse erythrocytes by VMH progressed in three stages: binding to an erythrocyte membrane, formation of a transmembrane pore, and explosion of the cell membrane. This hemolytic process is very similar to that of *Vibrio vulnificus* hemolysin (10, 41). However, their hemolytic kinetics were

TABLE 1. Purification of VMH

Purification step	Vol of purifying solution (ml)	Total amt of protein recovered (mg)	Total hemolytic activity (HU)	Sp act (HU/mg)	Relative activity	Yield of activity (%)
Collection of culture supernatant	2,400	12,300	456,000	37.1	1.0	100.0
Ammonium sulfate fractionation	20	450	250,000	558	15.0	54.8
Phenyl Sepharose column chromatography	1.5	2.2	165,000	75,000	2,020	36.2
Superose 6 column chromatography	2.0	0.3	31,000	103,000	2,790	7.2

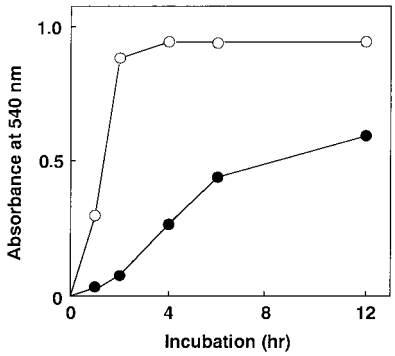

FIG. 2. Hemolysis induced by VMH dissociated from horse erythrocyte ghosts. The hemolysin (2 HU) was completely associated with horse erythrocyte ghosts (20 or 50 mg) by incubation at 37°C for 30 min in a total of 1 ml of Tris-HCl containing 0.9% NaCl. Thereafter, 1 ml of 1% intact horse erythrocytes was added. The admixture was incubated at 37°C, and the increases in A_{540} due to the liberation of hemoglobin from disrupted erythrocytes were measured periodically. Symbols: ○, hemolysis by VMH dissociated from 20 mg of horse erythrocyte ghosts; ●, hemolysis by VMH dissociated from 50 mg of horse erythrocyte ghosts.

significantly different from each other. As described by Gray and Kreger (10), hemolysis by *V. vulnificus* hemolysin terminated within a few hours and the rate of hemolysis depended upon the amount of the hemolysin. For instance, 2 HU of *V. vulnificus* hemolysin induced 100% hemolysis while the hemolytic reaction induced by 0.5 HU of the hemolysin terminated at 60% hemolysis. By contrast, the hemolytic reaction induced by VMH continued for a long period. Thus, all erythrocytes in the assay system were hemolyzed by prolonged incubation, even when an amount of VMH as small as 0.25 HU was allowed to act on the erythrocytes. These findings may demonstrate reversibility of the binding of the VMH molecule to the erythrocyte membrane. In order to verify this point, we carried out the following experiments.

VMH (2 HU) was associated preliminarily with horse erythrocyte ghosts (20 or 50 mg) by incubation at 37°C for 30 min, and intact horse erythrocytes were then added to the preparation of the VMH-ghost cells. When the admixture was allowed to incubate at 37°C, the steady lysis of horse erythrocytes by VMH dissociated from the ghost cells was observed in a time-dependent manner (Fig. 2). Next, we examined detachment of VMH molecules from the erythrocyte ghosts by incubation with gangliosides, putative components of the binding site for VMH on the erythrocytes (33). The erythrocyte ghosts, whose membranes carry 4 HU of VMH, were divided into two portions. One portion was incubated with 5 mg of gangliosides (mixed type; Sigma Chemical) at 37°C for 4 h, while another was incubated with saline. After incubation, the ghost cells were collected by centrifugation and the amount of VMH associated with the ghost cells was semiquantified by immunoblotting. The blotting showed that a significant amount of VMH was on the erythrocyte ghosts when incubated with saline for 4 h, but no protein band corresponding to VMH was detected when the erythrocyte ghosts were incubated with gangliosides (data not shown). This result suggests transfer of VMH molecules from erythrocyte membranes to the extracellular affinitive materials, i.e., gangliosides.

With these data being considered together, it may be concluded that VMH binds reversibly to the erythrocyte membrane so that this hemolysin causes a continuous hemolysis. Such a continuous hemolytic phenomenon in VMH is not uncommon. The thermostable direct hemolysin and its homologs produced by enteropathogenic vibrios were also reported to disrupt mammalian erythrocytes by the same kinetics (42).

Although VMH evidently induced an incessant hemolysis, the following results indicated that this hemolysin was unlikely to be a phospholipase C. When as small a dose as 4 HU of *Clostridium perfringens* alpha-toxin (Sigma Chemical), a prototype of bacterial hemolytic phospholipase C, was allowed to act on 0.7% phosphatidylcholine at 37°C for 4 h, a sufficient hydrolysis of the substrate was observed by the method of Geoffroy et al. (9). However, the purified VMH showed no lipolytic activity even when 1,000 HU of the toxin was allowed to act on the substrate.

Inhibitory effects of divalent cations. With several vibrio hemolysins, the hemolytic reaction has been documented to be inhibited by divalent cations, such as Ca^{2+}, Mg^{2+}, and Mn^{2+} (23, 29, 32, 45). The mechanism(s) by which the divalent cations inhibit the hemolytic reaction is poorly understood. However, Park et al. (29) recently reported that a transmembrane pore formed by *V. vulnificus* hemolysin was impermeable to Ca^{2+}, so that this cation possibly functions as an osmotic protectant which protects from the increase in the intracellular osmotic pressure through blockage of influx of extracellular water via the pore.

In order to study the effects of divalent cations on hemolysis by VMH, the toxin (2 HU) was allowed to act on horse erythrocytes at 37°C for 1 h in the presence of various concentrations (0 to 30 mM) of the cation. Thereafter, the extents of both hemoglobin release, which indicates cell explosion, and K^+ efflux, which indicates pore formation, were measured as described previously (16, 41). The 50% inhibitory doses (ID_{50}s) were estimated and are presented in Table 2. The hemolysis was apparently inhibited by all of the divalent cations tested, but the cations were able to be subdivided into two categories on the basis of their inhibitory potentials. Specifically, Cu^{2+}, Zn^{2+}, and Ni^{2+} showed inhibitory effects on both hemoglobin release and K^+ efflux at concentrations of less than 1.0 mM, but hemoglobin release was inhibited more efficiently. On the other hand, other cations, including Ca^{2+}, inhibited only hemoglobin release at concentrations of around 10 mM.

The following findings demonstrated that none of the divalent cations bound irreversibly to VMH or horse erythrocyte membranes. Even though VMH was treated with an inhibitory amount of each of the cations at 37°C for 30 min, full activity was recovered by dialysis against the assay buffer. In the same

TABLE 2. Inhibitory effects of divalent cations on the hemolytic reaction induced by VMH[a]

Cation[b]	ID_{50} (mM) for:	
	Hemoglobin release[c]	K^+ efflux[d]
Cu^{2+}	0.02	0.09
Zn^{2+}	0.07	0.30
Ni^{2+}	0.09	0.54
Mn^{2+}	6.8	30.0
Mg^{2+}	11.5	—[e]
Ca^{2+}	15.5	—

[a] VMH (2 HU) and horse erythrocytes (0.5%) were incubated at 37°C for 1 h in the presence of the cation. Thereafter, hemoglobin release and K^+ efflux were measured and the ID_{50} values were estimated.
[b] Chloride salts were used.
[c] Hemoglobin was quantified by measuring absorbance at 540 nm.
[d] The K^+ ion was quantified with a K^+-specific electrode.
[e] No inhibitory effect was observed with the cation at 30 mM.

TABLE 3. Number of VMH molecules bound to an erythrocyte membrane at 50% hemolysis[a]

Temp	Erythrocyte type	VMH added (molecules/cell)	VMH bound (molecules/cell)	VMH bound/ VMH added
37°C	Horse	1,800	1,800	1.00
	Sheep	3,800	2,800	0.74
	Human	39,000	25,000	0.64
25°C	Horse	4,600	3,600	0.78
	Sheep	41,000	8,600	0.21
	Human	41,000	12,000	0.29

[a] VMH, which caused 50% hemolysis after 30 min of incubation, was allowed to act on each of the erythrocytes at 25 or 37°C for 30 min in the presence of 30 mM dextran 4, an osmotic protectant. After incubation, the reaction mixture was centrifuged, the supernatant was collected, and the amount of free VMH in the supernatant was determined. Thereafter, the number of cell-bound VMH molecules was estimated.

TABLE 4. Characterization of the binding site for VMH on mammalian erythrocyte membranes[a]

Erythrocyte type	Result at 37°C		Result at 25°C	
	K_d (M, 10^{-8})	B_{max} (molecules [10^5]/cell)	K_d (M, 10^{-8})	B_{max} (molecules [10^5]/cell)
Horse	1.1	2.3	2.5	1.9
Sheep	1.1	1.1	2.5	0.9
Human	1.1	1.1	2.5	0.9

[a] VMH (0 to 4.5 pmol) was allowed to act on each type of erythrocyte at 25 or 37°C for 30 min in the presence of 30 mM dextran 4. After incubation, the reaction mixture was centrifuged, the supernatant was collected, and the amount of free VMH in the supernatant was determined. Thereafter, the amount of cell-bound VMH was estimated, and the values for K_d and B_{max} were calculated by Scatchard plot analysis.

manner, the cation-treated horse erythrocytes also recovered sensitivity to VMH by being washed with the buffer.

Different susceptibilities of mammalian erythrocytes to VMH. Our preliminary experiments demonstrated that although various types of mammalian erythrocytes were susceptible to VMH, their sensitivities were different from each other. At 37°C, about one-half of the horse erythrocytes in the assay system were hemolyzed with as small a dose as 10 ng of VMH within 1 h, but most of the human erythrocytes were not disrupted by incubation with 100 ng of the toxin for 1 h. On the other hand, sheep erythrocytes showed an intermediate level of susceptibility at 37°C and were most resistant to the hemolysin at 25°C. Because VMH formed small pores with identical pore sizes (ca. 3 nm) on all of the types of erythrocytes tested (data not shown), the difference in their susceptibilities was thought to be due to different levels of progress in the toxin-binding and/or pore-forming stage(s). In order to find the critical factor(s), a series of experiments were carried out as described below.

An amount of VMH causing 50% hemolysis after a 30-min incubation was allowed to act on horse, sheep, or human erythrocytes at 25 or 37°C for 30 min. Thereafter, the amount of VMH not bound to the erythrocytes was quantified and the amount of cell-bound VMH was estimated. The numbers of toxin molecules that bound to single erythrocyte cells of the three types of mammals are shown in Table 3. At either reaction temperature, horse erythrocytes were found to adsorb the toxin most efficiently and to be disrupted by the least amount of cell-bound toxin molecules. Hence, VMH attaches to and forms transmembrane pores on horse erythrocytes more easily than with sheep or human erythrocytes.

The efficient toxin binding described above may suggest that the binding site for VMH on horse erythrocyte membranes is different from those on other types of membranes in nature. In order to characterize the toxin-binding site, various amounts (0 to 4.5 pmol) of VMH were allowed to act on each type of mammalian erythrocyte at 25 or 37°C for 30 min and amounts of VMH that bound to the erythrocytes were determined. Thereafter, the K_d (dissociation constant of VMH) values and the numbers of binding sites (B_{max}) were calculated by Scatchard plot analysis. As shown in Table 4, horse erythrocyte membranes evidently had double binding sites at either temperature, whereas the K_d of the toxin to the horse was equal to those to other types of erythrocytes. Therefore, it may be concluded that VMH can associate readily with horse erythrocytes because they have higher numbers of binding sites than sheep and human erythrocytes. Incidentally, the estimated K_d of VMH was very close to that of the thermostable direct hemolysin (43), which also induces an incessant hemolysis.

Enterotoxicity of VMH. The pore-forming bacterial hemolysins isolated from human enteropathogens have been documented to have enterotoxic activity (15, 30). Similarly, VMH induced significant accumulation of bloody fluid in a dose-dependent manner when more than 50 μg of VMH was inoculated into a ligated ileal loop. However, the upper region of the small intestine was slightly sensitive to the hemolysin. It should be noted that the observed fluid-accumulating ability of purified VMH was comparable to those of *V. cholerae* hemolysin (15) and *V. parahaemolyticus* thermostable direct hemolysin (24).

Strain E-33 is an enteropathogenic strain (3). Thus, living cells, but not Formalin-killed cells, elicited bloody fluid accumulation at 18 h postinjection (Fig. 3). The enteropathogenicity of this bacterium was apparently reduced by the simultaneous injection of the antibody against VMH (Fig. 3). This strongly suggests that VMH is an important virulence determinant of *V. mimicus* E-33, which may cause dysentery-like diarrhea.

DISCUSSION

Studies of kinetics of the hemolytic reaction demonstrated that VMH induces continuous hemolysis. Many bacterial pore-forming toxins have been reported to bind in a monomeric form to an erythrocyte membrane, but the toxin molecules

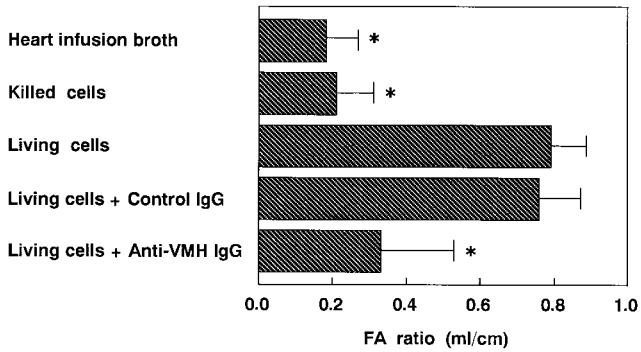

FIG. 3. Fluid accumulation by *V. mimicus* cells and its inhibition by the antibody against VMH. The bacterial cells (10^8 cells in 0.5 ml of fivefold-diluted heart infusion broth) were mixed with 0.5 ml of saline, anti-VMH IgG, or control IgG. Each of the mixtures was injected into a ligated ileal loop, and fluid accumulation (FA) was measured at 18 h postinjection. Each value represents the mean ± standard deviation of four experiments. *, $P < 0.01$ versus the value for living cells alone or living cells plus control IgG.

autoassemble on the membrane to construct a transmembrane pore (1). The binding of the monomer toxin streptolysin O from *Streptococcus pyogenes* A was recently described as reversible, while that of the assembled oligomer toxin was described as irreversible (38). It is not known whether VMH molecules also autoassemble on the erythrocyte membrane. However, even though VMH forms an oligomer on the membrane, it seems to dissociate from the erythrocyte membrane and to attack in a monomeric or oligomeric form another intact erythrocyte.

Like other vibrio hemolysins (23, 29, 32, 45), the hemolytic reaction induced by VMH was inhibited by various divalent cations. Although the pore-forming stage was also inhibited by some cations, including Zn^{2+}, at high concentrations, the cell-exploding stage was inhibited more effectively by all divalent cations tested. Therefore, the inhibition of this final hemolytic stage is considered to be the prime action of each divalent cation. In contrast to *V. vulnificus* hemolysin (29), VMH was found to form a pore permeable to divalent cations (data not shown). Hence, the cations may act inside the erythrocyte. The activation of transamidase, causing the cross-linking of erythrocyte membrane proteins (20) and/or the stabilization of the membrane skeletal network mediated by the divalent cation spectrin (39), is the possible inhibitory mechanism(s) of the divalent cation. Like some pore-forming toxins (19, 23), Zn^{2+} inhibited strongly a pore-forming stage of VMH. The extracellular inhibitory effect of Zn^{2+} may be due to stabilization of the lipid bilayer through direct contact with phospholipids (26, 34). However, the precise extracellular inhibitory mechanism of the cation is not yet definitively understood.

VMH may hemolyze many kinds of mammalian erythrocytes. However, of the three types of erythrocytes studied, horse erythrocytes were the most sensitive to lysis by VMH because they had the most toxin-binding sites and were disrupted by the least amount of membrane-associated toxin molecules. The increase in the level of membrane fluidity has been documented to be a critical determinant in the formation of transmembrane pores and to cause hemolysis (1, 36). Because the membrane fluidity of mammalian erythrocytes is modulated by membrane enzymes inducing phosphorylation of membrane proteins (22) and/or hydrolysis of membrane phospholipids (31), the enzyme(s) in horse erythrocyte membranes may be activated more effectively than in other types of erythrocytes by attachment of the VMH molecule to the membrane. A study to find a target enzyme(s) triggered by the binding of VMH is in progress. The erythrocytes from sheep were hemolyzed readily at 37°C but were less sensitive to lysis at 25°C. Sheep erythrocyte membranes are known to contain a high percentage of sphingomyelin. An increase in the proportion of sphingomyelin is concomitant with a decrease in membrane fluidity, especially at low temperatures (2). Accordingly, a decrease in membrane fluidity may be a predominant determinant causing the reduced pore formation on sheep erythrocyte membranes at 25°C.

Except for some isolates, virulent strains of *V. mimicus* lack the ability to produce so-called enterotoxin (4, 6, 27). Living cells of strain E-33, an enterotoxin-negative strain (3), elicited accumulation of bloody fluid when they were inoculated into a ligated rabbit ileal loop. This finding indicates that this strain has the active principle(s) stimulating and/or damaging intestinal mucosal cells. Purified VMH also showed the ability to accumulate bloody and mucous fluid in the ileal loop. Additionally, the IgG antibody against the hemolysin obviously abolished fluid accumulation by the bacterium. These findings strongly suggest that VMH is involved in enteropathogenicity of *V. mimicus* E-33. The hemolysins from several enteropathogenic vibrios have enterotoxic activities (15, 30), but the mechanisms of action have been poorly studied. The mechanism by which VMH controls enterotoxicity is also not clear. Further experiments using Ussing chambers and/or tissue cultures will clarify this point.

In the present study, we purified an enterotoxic 63-kDa hemolysin from a virulent strain of *V. mimicus* and found that the hemolysin induced a continuous hemolysis because of reversible binding to erythrocyte membranes.

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