Fluid accumulation in Mouse Ligated Intestine Inoculated with the Vascular Permeability Factor Produced by *Bacillus cereus*

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ABSTRACT. Partially purified vascular permeability (VP) factor (VPF) of *Bacillus cereus* induced fluid accumulation in the ligated intestinal loops of mouse (MIL) and rabbit (RIL), suggesting that the VP activity may correlate with fluid accumulation in ligated intestinal loops of these animals. Fluid accumulation was observed at 6–8 hr in 55–67% of mouse intestinal loops inoculated with 40–50 immunodiffusion units (IDU) of partially purified VPF, whereas it was found at 2 hr in all loops with 400–600 IDU of partially purified VPF. In rabbit intestinal loops with 120–190 IDU of partially purified VPF, fluid accumulation was observed at 6 hr. From these findings, VPF produced by *B. cereus* can be easily detected in both MIL and RIL. The intestinal tissue of mouse intestinal loops was histopathologically damaged at different concentrations of the VPF to induce fluid accumulation. With 50 IDU of partially purified VPF, severe edema was found in the lamina propria layer and submucosa. With 600 IDU of partially purified VPF, on the other hand, severe necrosis in the surface epithelium of villus and lamina propria layer, and hyperemia in the submucosa were observed, suggesting that partially purified VPF may be cytotoxic and/or intestinecrotic.—**KEY WORDS:** *Bacillus cereus*, fluid accumulation, mouse intestinal loop assay, vascular permeability factor.

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Bacillus cereus is known as an etiological agent to cause two types of food-borne gastroenteritis characterized by either diarrhea or vomiting [5, 6, 16]. Attempts have been made in recent years to demonstrate extracellular proteaceous substance(s) involved in gastroenteritis caused by *B. cereus* [13, 15–17]. Culture filtrates of *B. cereus* cause fluid accumulation in rabbit ileal loop (RIL) assay [13] and increase vascular permeability (VP) in rabbit skin [7] like other enterotoxins produced by *Vibrio cholerae* [1, 2], enterotoxigenic *Escherichia coli* [4], and *Clostridium perfringens* [3, 10]. The VP activity of *B. cereus* culture filtrate correlated with the RIL activity [7, 17]. The VP activity has been more commonly measured to determine enteropathogenicity of culture filtrate of *B. cereus* since it is simple to perform [5, 7, 16, 17]. However, the VP test alone has been shown to mislead to the results on enteropathogenicity of *B. cereus* culture filtrates since lecithinase, hemolysins, and proteinases in culture also showed VP activities [7, 15]. As to enterotoxins produced by enterotoxigenic *E. coli* [13] and *C. perfringens* [19], mouse intestinal loop assay (MILA) have been developed to more easily determine the enteropathogenicity. To see whether MILA is applicable to determine enteropathogenicity of culture filtrate of *B. cereus*, therefore, the

present study was undertaken to examine enteropathogenicity of vascular permeability factor (VPF) produced by *B. cereus* in ligated intestinal loops of mice.

MATERIALS AND METHODS

Bacterial strain and preparation of partially purified VPF: *B. cereus* strain FM-1, originally isolated from a diarrheal-type food poisoning outbreak, was used to prepare VPF. For VPF production, a fresh brain heart infusion (BHI) agar slant (Difco Laboratories, Detroit, Mich., U.S.A.) culture, incubated for 12 hr at 32°C, was used to inoculate 500-ml Sakaguchi-flask containing 50 ml of BHI broth. After shaking (100 rpm) for 14–16 hr at 32°C, 10 ml of the seed culture was transferred to 1,000 ml of BHI broth supplemented with 10 g of glucose (BHIG) contained in 3-liter Sakaguchi-flask. The culture was incubated with shaking (100 rpm) for 5–6 hr at 32°C. Bacterial cells were removed by centrifugation at 8,000 rpm for 20 min at 4°C. According to the methods of Spira and Goepfert [14], solid ammonium sulfate was added to the culture supernatant to 70%. The precipitate formed was collected by centrifugation and dissolved in 50 ml of 5 mM Tris-HCl, pH 8.6. The

suspension was extensively dialyzed against the same buffer at 4°C. The clear supernatant obtained by centrifugation was concentrated by lyophilization and then fractionated by gel filtration on Sephadex G-100 (2.5 × 95 cm) equilibrated with the same buffer. The fractions containing the VP activity were collected and used as partially purified VPF. The VPF was about 50-fold purified from culture supernatant on a protein basis.

Biological assays: The VP activity and the ligated intestinal loop tests using rabbits and mice were carried out by the procedure described previously [12]. Rabbits and mice were anesthetized by intraperitoneal injection of sodium pentobarbital (200 mg/kg of rabbit and 600 µg/mouse, respectively) and the abdomen was opened along the midline. Ligations were made at 7–8 cm from the jejunum and spaced to yield 6 test loops per rabbit: the test loops were separated by interloops of approximately 2.5–3 cm in length. In mice, on the other hand, ligations were made at the upper part of the jejunum to yield one loop per mouse. One and 0.1 ml of the test sample was administered into each loop of rabbit and mouse, respectively. Animals were held for 2, 4, 6, 8, or 10 hr postsurgery before they were sacrificed. Fluid accumulated in each loop was withdrawn to be measured and each loop was then measured to determine the exact length. A positive response was determined as a volume(ml)/length (cm) ratio greater than or equal to 0.5.

Histopathological studies: At timed intervals the loops of mice were removed, cut open longitudinally, fixed overnight in 10% formaldehyde solution, embedded in paraffin, and sectioned transversely at 5 µm. Sections were stained with hematoxylin and eosin (HE staining) according to McDonel and Duncan [9].

Preparation of antiserum and immunological methods: Six hundred microliters (5–100 µg) of partially purified VPF was emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich, U.S.A.) and intraperitoneally injected into rabbits. Injections were made on days 0, 7, 14, and 21. Two weeks later, the rabbits were boosted with partially purified VPF (100 µg) emulsified in Freund's incomplete adjuvant. The antigenicity of partially purified VPF was determined by microslide gel diffusion. One immunodiffusion unit (1 IDU) of partially purified VPF was defined as the reciprocal of the highest dilutions to form the precipitin line(s) against rabbit

antiserum reactive with partially purified VPF at 6 µg/ml.

RESULTS

Dose response and time course of fluid accumulation: The time-course of fluid accumulation in the ligated intestinal loops of mice in response to different amounts of partially purified VPF are summarized in Table 1. Fluid accumulation was very rarely found in all of intestinal loops of mice inoculated with 20 IDU of partially purified VPF (Table 1). When inoculated with 40–50 IDU of the VPF, mice showed fluid accumulation in 67% and 55% of the loops tested at 6 and 8 hr, respectively (Table 1). With 500 IDU of VPF, fluid accumulation was found in almost all loops at 4–6 hr after inoculation, whereas it was found in less loops at 8 hr or longer. With 400–600 IDU of partially purified VPF, fluid accumulation was found in 100% and 75–90% of the loops tested at 2 hr and 4–10 hr, respectively (Table 1). With inoculation of 120 IDU of partially purified VPF, on the other hand, fluid accumulation was found in 18, 9, and 0% of the loops tested at 2, 6 and 8–10 hr, respectively (Table 1).

Similar intestinal loop tests were also performed in rabbits with partially purified VPF (Table 2). No fluid accumulation was found in rabbit ligated intestinal loops inoculated with 60 or 90 IDU of partially purified VPF at 6 and 10 hr (Table 2). On the other hand, fluid accumulation was found in 60–80% of the ligated intestinal loops inoculated

Table 1. The time-course of the appearance of fluid accumulation in the mouse ileal loops

Amount of vascular permeability factor (IDU) ^{a)}	Incubation time (hr)				
	2	4	6	8	10
20	0/5 ^{b)} (0)	1/9 (11)	1/19 (5)	1/4 (25)	0/4 (0)
40–50	3/11 (27)	5/13 (39)	16/24 (67)	6/11 (55)	5/11 (46)
120	2/11 (18)	1/16 (6)	2/23 (9)	0/10 (0)	0/4 (0)
400–600	6/6 (100)	4/5 (80)	7/8 (88)	4/5 (80)	3/4 (75)

a) IDU, immunodiffusion unit: the minimal amount of partially purified vascular permeability factor (VPF) to form precipitin line(s) against rabbit anti-partially purified VPF.

b) Number of loops with fluid accumulation/Total number of loops tested, Number in parentheses: percent.

Table 2. Fluid accumulation in the rabbit ileal loops inoculated with different amounts of partially purified vascular permeability factor

Incubation time (hr)	Amount of partially purified vascular permeability factor (IDU) ^{a)}					
	60	90	120	190	380	600
6	0/4 ^{b)}	0/3	3/5	3/3	3/3	3/3
10	0/4	0/3	4/5	3/3	3/3	3/3

a) IDU, immunodiffusion unit: the minimal amount of partially purified vascular permeability factor to form precipitin line(s) against rabbit anti-partially purified vascular permeability factor.

b) Number of loops with fluid accumulation/Total number of loops tested.

Table 3. Histopathological findings of mouse intestinal loops inoculated with different amounts of partially purified vascular permeability factor (VPF)

Incubation time (hr)	Amount of VPF (IDU) ^{a)}	Fluid accumulation	Surface epithelium	Proprial layer			Submucosa		
			Necrosis, Destruction	Edema	Necrosis	CI ^{b)}	Edema	Hyperemia Hemorrhage	CI
0.5	50	(-)	-	-	-	-	-	-	±
	120	(-)	-	-	-	-	-	-	-
	600	(+)	++	-	++	-	++	++	-
2	50	(-)	-	-	-	+	+	-	-
	120	(-)	-	-	-	+	-	-	-
	600	(+)	+++	±	+++	+	±	+++	-
6	50	(+)	-	+++	-	++	+	-	-
	120	(-)	-	-	-	+	-	±	-
	600	(+)	+++	++	+++	+	±	+++	-
10	50	(+)	-	++	-	+++	+++	+	-
	120	(-)	-	-	-	+	-	-	-
	600	(+)	++	+++	++	+	++	+++	-

-: Negative, ±: Rare, +: Slight, ++: Moderate, +++: Marked

a) IDU, immunodiffusion unit: the minimal amount of partially purified vascular permeability factor (VPF) to form precipitin line(s) against rabbit anti-partially purified VPF.

b) CI: Infiltration of neutrophils or plasma cells.

with 120 IDU of partially purified VPF, whereas that was observed in all of the loops inoculated with 190, 380, or 600 IDU of the VPF (Table 2).

Histopathological studies: To see whether the concentration of partially purified VPF to induce fluid accumulation in ligated intestinal loops of mice occur histological damage, histopathological examinations of the intestinal tissues in each loop were done at different periods after inoculation. The results obtained are summarized in Table 3.

Almost no histopathological damages of the intestinal tissues were found in each loop inoculated with 50 IDU of VPF until 2 hr after inoculation. At 6–10 hr after inoculation, however, severe edema was found in the lamina proprial layer and submucosa. Severe infiltration of neutrophils was also

found in the lamina proprial layer although necrosis was not observed (Table 3). Similar histopathological damages were weakly observed in the intestinal tissue of loops with no fluid accumulation induced by 50 IDU of VPF. On the other hand, severe necrosis and destruction in the surface epithelium of villus, necrosis in the lamina proprial layer, and hyperemia and hemorrhage in the submucosa were found at 2 hr in each loop inoculated with 600 IDU of VPF. Such histopathological damages of the intestinal tissue were observed more severely with longer exposure of VPF to each loop (Table 3). In contrast to these histopathological findings, almost no or very slight histopathological damages were found in each loop inoculated with 120 IDU of VPF.

DISCUSSION

Although RIL tests have been used to determine enteropathogenicity of culture filtrate of *B. cereus* [13], it is not easy to handle. Since the VP activity of culture filtrate of *B. cereus* has been shown to correlate with fluid accumulation in RIL tests [7, 17], the VP test is more commonly used to determine the enteropathogenicity [5, 15–17]. However, Thompson *et al.* [15] have recently shown that the VP tests alone may mislead to the results since lecithinase, hemolysins, and proteinases in culture filtrates also showed VP activities. The present study demonstrated that VPF produced by *B. cereus* induced distinct fluid accumulation in the ligated intestinal loops of mice and rabbits as found for other enterotoxins (ETs) of enterotoxigenic *E. coli* [11] and *C. perfringens* [19]. Thus, the present results suggest that the mouse intestinal loop test is easy to rear and handle and is valuable to determine the enterotoxic activity of culture filtrate of *B. cereus*.

Although variations in fluid accumulation were found in mouse intestinal loops inoculated with different amounts of partially purified VPF, fluid accumulation occurred was not dose-dependent. This is in contrast to other ETs produced by *V. cholerae*, enterotoxigenic *E. coli*, and *C. perfringens* since fluid accumulation caused by these ETs are dose-dependent [8, 11, 19]. With 40–50 IDU of partially purified VPF, mice showed fluid accumulation in 55–67% of the loops tested at 6–8 hr after inoculation. Fluid accumulation was most frequently found in intestinal loops of mice at 6–8 hr after inoculation, whereas that was found in intestinal loops of rabbit at 6–10 hr with 120–190 IDU of partially purified VPF. These results suggest that VPF produced by *B. cereus* can be detected in both mouse and rabbit ileal loop tests. However, an appropriate amount of VPF should be administered into the mouse intestinal loop when the assays is used to determine the enteropathogenicity since variation in fluid accumulation was observed in mouse intestinal loops with different amounts of partially purified VPF.

In histopathological studies, marked edema was observed at 6 hr in both lamina propria layer and submucosa of the loops inoculated with 50 IDU of partially purified VPF. On the other hand, severe necrosis and destruction in the surface epithelium,

necrosis in the lamina propria layer, and hyperemia in the submucosa were found at 2 hr or longer in the loops inoculated with 600 IDU or more of partially purified VPF. Turnbull *et al.* [17, 18] have reported that VPF produced by some strains of *B. cereus* showed dermonecrotic activity to rabbit skin, whereas it showed intestinecrotic activity and caused fluid accumulation in intestinal loops of animals. From these findings, they suggest a possibility that different amounts of the VPF may cause various biological activities to different animal tissue and/or organs. Thus, the possibility may partly account for variation in fluid accumulation in mouse intestinal loops with different amounts of partially purified VPF. To prove such possibility, purification of VPF produced by *B. cereus* will be needed.

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