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RESEARCH NOTE

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Toxicity of 5 *Bacillus cereus* Enterotoxins in Human Cell Lines and Mice

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Abstract To determine whether the toxicity of *Bacillus cereus* would be seen in human cell lines and mice, we screened *B. cereus* B-38B, *B. cereus* B-50B, and *B. cereus* KCCM40935 for genes that coded for 5 enterotoxins using the polymerase chain reaction and cultivated them for 17 hr, by whose time they had grown to 10^7-10^8 colony-forming units (CFU) per milliliter. Cell-free supernatant was added to make up 1% of the total reaction solution. Human cells from normal lung, lung carcinoma, embryonic kidney, and cervical adenocarcinoma cell lines were grown in culture. The cytotoxicity induced by adding the reaction solution was indicated by cell death rates of 0 to 70%, depending on the bacterial strain involved and the cell line. A lethality of 20% was observed when *B. cereus* cultures containing 10^7-10^8 viable cells were administrated orally to mice. Therefore, the culture of *B. cereus* containing 10^7-10^8 viable cells seems to have high cytotoxicity on human cell lines and lethality on mice.

Key words: *Bacillus cereus*, enterotoxins, SRB assay, cytotoxicity, mouse lethality

Introduction

The *Bacillus cereus* organism is found widely in foods and food products and has been known to cause food poisoning. Food-borne diseases caused by *B. cereus* are characterized by emesis or diarrhea. Emesis is caused by a single toxin, cereulide. Some strains produce heat-labile enterotoxins during the vegetative cell growth in the small intestine, which causes diarrhea (1-6). This organism causes diarrhea more often than emesis; for this reason, many studies have concentrated on the diarrheal enterotoxins. Four enterotoxins have been described for various *B. cereus* species: hemolysin BL (HBL), nonhemolytic enterotoxin (NHE), and 2 enterotoxins encoded by *entFM*, and *bceT* (7-10). Lately, however, cytotoxin K, which is closely related to the β-toxin of *Clostridium perfringens*, was detected in *B. cereus* (11) and reported to cause worse symptoms than the other toxins.

The majority of research on the toxic characteristics of *B. cereus* has focused on the enterotoxin HBL. This tripartite molecule contains 2 lytic components (L1 and L2) and a binding component (B) (9). A commercial visual immunoassay (VIA) system that is used to identify enterotoxins detected this 45 kDa toxin (12). NHE, the 3-component complex that was originally isolated and identified in *B. cereus* as for a causative organism in food-poisoning (13), has also been the subject of a considerable number of studies. Enterotoxin FM, enterotoxin T (14), and CytK (11) are also thought to result in food poisoning due to *B. cereus* exposure. However, their structures and toxicity have not been well characterized. It has been reported that *B. cereus* intake of 10^7-10^8 cells/g food is needed to induce diarrhea and 10^6-10^7 cells/g to induce emesis. However, there is no clear quantitative guideline for identifying a specific toxic dose.

We isolated *B. cereus* species that have the genes for all 5 of these enterotoxins using the polymerase chain reaction (PCR). Cell-free culture supernatant containing these toxins was administered to the human cell line cultures and to mice in order to gain information about the cytotoxicity and the lethality by *B. cereus*.

Materials and Methods

Strains and cultivation *B. cereus* B-38B, *B. cereus* B-50B, and *B. cereus* KCCM 40935 were selected from our laboratory collection (15). Each strain contained genes for the 5 enterotoxins: *hbl*, *nhe*, *cytK*, *bceT*, and *entFM*. These organisms were originally isolated from raw rice grown on mannitol–egg-yolk–polymicin (MYP) agar containing 5% egg-yolk emulsion (Difco Laboratories, Detroit, MI, USA) and polymicin B 20,000 IU (Sigma Ltd, St. Louis, MO, USA). The rice was inoculated onto MYP agar plates, which were incubated at 37°C for 24-48 hr. Colonies with typical pink, opaque halos, were collected from the plates, underwent Gram staining, and were analyzed for catalase activity, hemolysis, and morphology.

The organisms were cultivated in a brain heart infusion broth (Difco Laboratories) for 17 hr and underwent centrifugation at 110 rpm at 37°C; the broth was then used as the test culture. To obtain a viable cell count, the culture was diluted with 0.85% saline, spread on the MYP agar, and incubated at 37°C for 24-48 hr.

Detection of hemolysin enterotoxin Hemolysin BL was detected using a Tecra kit (Tecra Diagnostics, Roseville, Australia) and an Oxoid kit (Oxoid Ltd, Hampshire, England) according to the manufacturers' instructions.

Measurement of toxicity in the human cell lines The 4 human cell lines included normal lung (L132), lung carcinoma (A549), embryonic kidney (293a), and the

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cervical adenocarcinoma (HeLa) cells. These cells were cultivated in Dulbecco's Modification of Eagle's Medium (DMEM; Invitrogen Corporation, CA, USA) containing 10% fetal bovine serum (FBV). A 200-μL sample containing 80-120 cells at the log phage was distributed among 96-well microtiter plates and left for 24 hr in an atmosphere of 5% CO_2 at 37°C to promote cell stability. *B. cereus* cultures containing cells in the stationary phase were centrifuged at 8,000×g for 10 min. The supernatant was filtered through a 0.45-μM membrane filter then diluted 1-, 5-, and 10-fold with phosphate buffer saline (PBS) after neutralization. A 2-μL sample of the diluent was added to each well and incubated for 72 hr. The medium in the wells was then removed completely. Trichloroacetic acid solution (10%) was added and left at 4°C for 1 hr. The well plates were washed 3 times with distilled water and then dried at room temperature. Sulforhodamine B (SRB; 0.4%) 50 μL was added to the well plates, which were then placed in the incubator for 1 hr (16, 17). The plates were washed 3 times with 1% acetic acid and dried. After the addition of 100 μL of tribase 10 mM, the plates were shaken for 5 to 10 min and their absorbance at 520 nm was measured by spectrophotometer (Tecan Sunrise™, Mnnedorn, Switzerland). Distilled water was used as a negative control instead of the culture supernatant.

Measurement of toxicity in mice Forty-five mice (CBA; age: 6 week) from the Yonsei Medical Center were given free access to food and drinking water. They were fed a commercial pellet chow and allowed to adapt for 5 days to a room with controlled temperature (23-25°C) and light (alternating 12-hr periods of light and dark). The mice were then divided into 9 groups of 5 mice each to receive an intraperitoneal (0.5 mL), intravenous (0.1 mL) and oral (1.0 mL) dose from the 17-hr culture. Lethality was calculated 24 hr later.

Results and Discussion

Screening and growth of *B. cereus* The typical pink colonies of 84 *B. cereus* isolates were confirmed by MYP and PCR assays for the genes for the 5 toxins, according to a technique described previously (15). The *B. cereus* B-38B and *B. cereus* B-50B strains with the genes for all 5 enterotoxins were selected for this study.

The Tecra and Oxoid kits were used to confirm the expression of the *hbl* (Table 1). Consequently, hemolysin production was detected, but production of the remaining 4 enterotoxins was not confirmed by gene expression. The growth curves for *B. cereus* KCCM40935, *B. cereus* B-38B, and *B. cereus* B-50B were determined in the brain heart infusion broth. When they reached the logarithmic growth phase, the cell number was 10^6-10^8 CFU/mL. The stationary phase started after about 6-7 hr, when 10^7-10^8 CFU/mL were present (data not shown). The cell number remained at this level throughout the 17 hr of incubation.

Cytotoxicity of the supernatant of the *B. cereus* culture in human cells When the cytotoxicity of *B. cereus* KCCM 40935, *B. cereus* B-38B, and *B. cereus* B-50B in L132 cells was measured, the nondiluted supernatant of *B. cereus* B-50B showed the highest toxicity (cell death rate: 70%), and the cell death rate was similar for the other *B. cereus* strains (60%). The diluted supernatant of *B. cereus* KCCM40935 and *B. cereus* B-50B demonstrated limited toxicity, but cytotoxic effects were seen with the supernatant of *B. cereus* B-38B. When the supernatants were diluted, their absorbances at 520 nm were similar (approx. 0.10-0.12). When their cytotoxicity was evaluated in A549, the supernatant of *B. cereus* B-50B had the least toxic effect, with cell death rates of 40 and 70% for *B. cereus* KCCM 40935 (40%) and *B. cereus* B-38B (70%), respectively, being dramatically higher. However, no cytotoxic effect was detected from the diluted supernatants of these species. When their cytotoxicity in 293a was evaluated, *B. cereus* B-50B was found to have a death rate of 60%; the cell death rates were lower for the nondiluted supernatant of the *B. cereus* B-38B and *B. cereus* KCCM 40935 cultures than for the *B. cereus* B-50B culture. In the HeLa line, the cell death rate for the nondiluted supernatants of these species was higher (60%) than for *B. cereus* B-50B (40%).

Generally, *B. cereus* B-50B demonstrated the lowest cytotoxicity in these cell lines and *B. cereus* B-38B demonstrated the highest cytotoxicity. The pattern of cytotoxicity for *B. cereus* B-50B was almost the same as that for *B. cereus* KCCM 40935. It seemed that the *B. cereus* supernatants had different concentrations of enterotoxin, even though they had the same genes for each toxin. One theory for this observation is suggested by reports that different levels of enterotoxin are released into the extracellular environment (i.e. the supernatant) by different species of *B. cereus* (18).

Lethality of *B cereus* culture in the mouse Nondiluted culture medium samples were injected intraperitoneally (0.5 mL) into one group of mice, intravenously (0.1 mL)

Table 1. Detection of enterotoxins of *B. cereus* and their genes using the polymerase chain reaction and commercial kits

Strains	Target toxin genes[1] and enterotoxin						
	hblA	nheA	cyt K	bceT	entFM	Oxoid kit[2]	Tecra kit[3]
B cereus KCCM40935	+	+	-	+	+	+	++
B cereus B-38B	+	+	+	+	+	+	++
B cereus B-50B	+	+	+	+	+	+	++

[1]+, PCR product was detected. These results were cited by Jang (15) and confirmed here again.
[2]+, agglutination pattern = hemolysin enterotoxin detection; -, non agglutination pattern.
[3]Color comparator for TECRA Visual immunoassays +, Positive 3; ++.

Table 2. Lethality of *B. cereus* after peritoneal, intravenous, and oral administration in mice

Microorganisms	Number of mice (dead/total)		
	1st group (Peritoneal injection)	2nd group (Intravenous injection)	3rd group (Oral administration)
B. cereus KCCM40935	0/5[1)]	0/5	1/5[2)]
B. cereus B-38B	0/5	0/5	1/5
B. cereus B-50B	0/5	0/5	1/5

[1)]No response by injection.
[2)]One dead mouse among five mice.

into the second group, and orally (1.0 mL) into the third group. The lethality in these 3 groups was observed after 24 hr. At that time, no lethal effect was found in the first and the second groups, but 1 in 5 mice of the third group was dead after 24 hr of exposure to each *B. cereus* species (Table 2). Although this death rate is low, it does indicate that the toxin had been released into the culture (19, 20).

B. cereus is distributed broadly in nature and has not been recognized as an important food-borne pathogen. However, the number of outbreaks of food poisoning on exposure to this organism has increased so rapidly (21) that Korean governmental agencies have tried to enforce specific regulations to prevent the contamination of food with this organism. While *B. cereus* may be present in a range of foods in low concentrations (< 10^3 CFU/g), it generally does not cause illness, unless time and temperature allow it to grow to numbers in the range of >10^5-10^6 CFU/g. *B. cereus* levels are not permitted by law to exceed 10^2 CFU/g in infant formula, because it can rise to 10^4 CFU/g when in the temperature rises. However, there is almost no toxicological data to determine guidelines for the safety by this organism.

In this study, we isolated the strains of *B. cereus* B-38B and *B. cereus* B-50B that have the genes that express all 5 enterotoxins found in these organisms to determine their toxicity in human cell lines. The human cell lines selected are usually used in tests of antibiotic substances (22). The highest toxicity was reported for *B. cereus* B-38B, which have been isolated from unhusked rice. To detect the cell death rate, the reaction solution had to contain 1% nondiluted supernatant. Only the nondiluted supernatants contained enough enterotoxin to have a toxic effect (indicated by the rate of cell death), and that effect appeared to depend on the bacterial concentration and the cell line. Almost no cytotoxicity was found for supernatants that had been diluted 5- or 10-fold.

The lethality in mice was detected by administrating 1.0 mL of the culture containing a bacterial concentration of 10^7-10^8 CFU/mL. We found that an adsorption of 10^7-10^8 cells and enterotoxins was lethal for the mouse. Little information was provided regarding the risk assessment for *B. cereus*; thus, the exact dose of *B. cereus* that conferred lethality is not known. Further quantitative

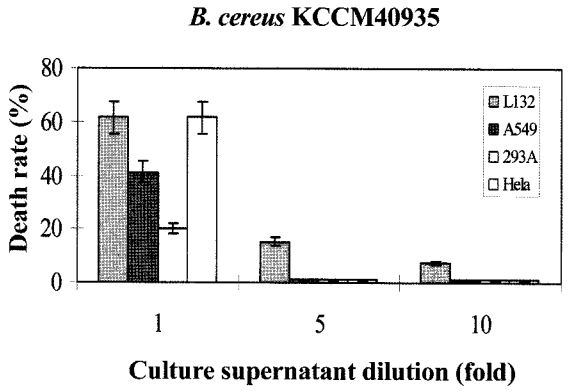

Fig. 1. Cytotoxicity of the supernatant of the *B. cereus* KCCM40935 culture on the cells from the human lung (L132), lung carcinoma (A549), embryonic kidney (293a), and cervix adenocarcinoma (HeLa) cell lines.

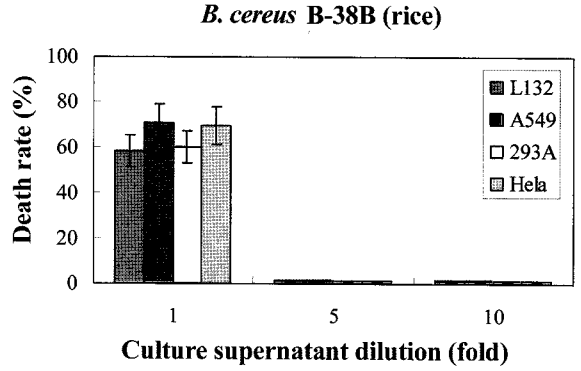

Fig. 2. Cytotoxicity of the supernatant of the *B. cereus* B-38B culture on human lung (L132), lung carcinoma (A549), embryonic kidney (293a), and cervix adenocarcinoma (HeLa) cell lines.

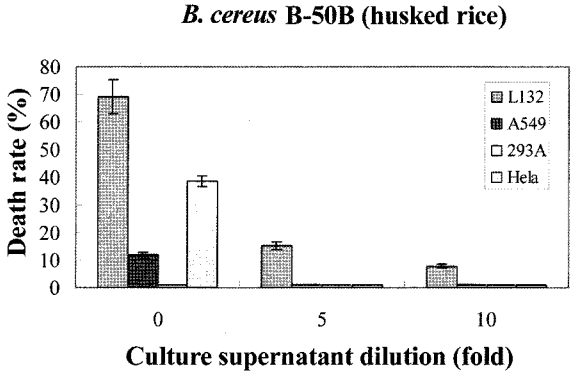

Fig. 3. Cytotoxicity of the supernatant of the *B. cereus* B-50B culture on human lung (L132), lung carcinoma (A549), embryonic kidney (293a), and cervix adenocarcinoma (HeLa) cell lines.

analyses with the purified enterotoxin will have to be carried out to determine safe levels of this organism in foods.

Acknowledgments

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