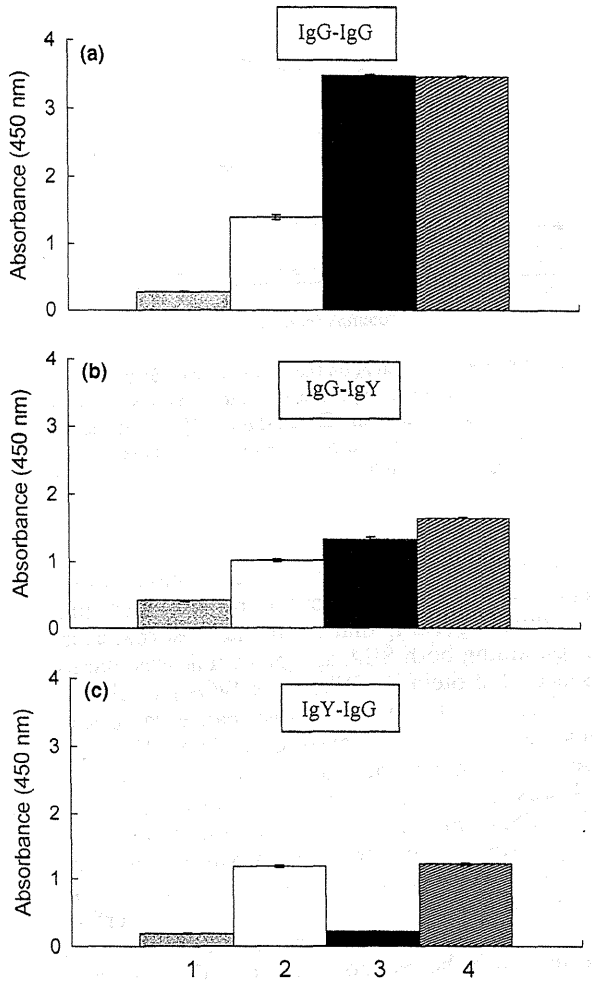

Figure 2 Effects of protein A on the absorption of the double-antibody sandwich ELISAs. IgG (Panels a and b) and IgY (Panel c) antibodies at 0.1 μg per well were coated in each well of ELISA plate. Column 1 : None, 2 : rSEA 1000 ng mL^{-1}, 3 : Protein A 1000 ng mL^{-1}, and 4 : rSEA 1000 ng mL^{-1} and Protein A 1000 ng mL^{-1}.

Determination of the concentration of Staphylococcal enterotoxin A using the ELISAs

Using the calculation curve shown in Fig. 4, the concentration of SEA in the supernatant cultured with sea gene positive *S. aureus* strains (196E and 11689) was determined using an IgG-IgG or IgY-IgG ELISA. When the IgG-IgG ELISA was used, a 10 000-fold dilution of the supernatant was necessary to adapt the calculation curve. In the case of the IgY-IgG ELISA, only a 300-fold dilution of the supernatant was necessary. The SEA concentration in the supernatants determined using IgG-IgG ELISA was dramatically higher than that

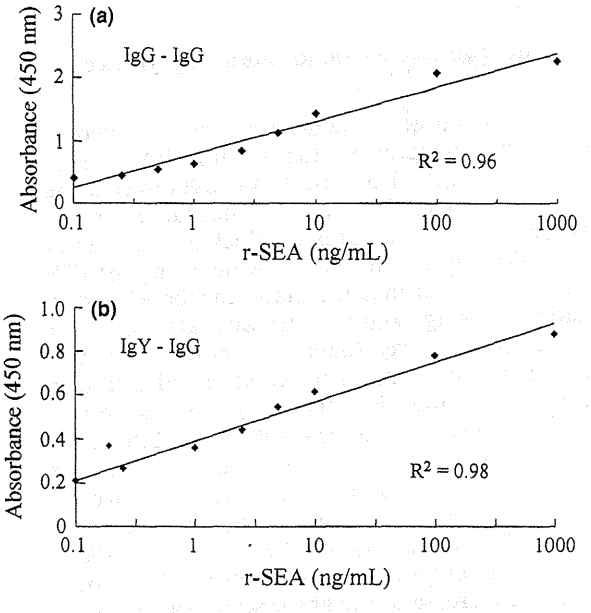

Figure 3 Calculation curve for determination of Staphylococcal enterotoxin A in the culture supernatant using the IgG-IgG (Panel a) and IgY-IgG (Panel b) ELISAs.

determined using the IgY-IgG ELISA (Table 1). The supernatant cultured with the strains lacking the *sea* gene (FRI-361, Saga-1 and Aomori 1) revealed an absorbance equal to that of the blank wells using the IgY-IgG ELISA, but that showed a markedly higher absorbance in the IgG-IgG ELISA (data not shown).

Discussion

Staphylococcal enterotoxin A is a protein responsible for staphylococcal food poisoning. Several immunoassays have been described for the detection of the enterotoxins in food extracts and culture supernatant fluids (Freed *et al.*, 1982). The authors mentioned that Ig isolated from the serum of non-immunized rabbits can resolve the interference in the ELISA reactions, because protein A binds to normal IgG. This adsorption method with normal IgG has been available, but the detection limits will be compromised, because the test materials must be diluted with normal IgG. However, these methods are frequently hampered by false-positive results due to protein A, which is produced by most strains of *S. aureus*. The binding of protein A to the Fc region of the IgGs of many species can lead to false-positive results in the ELISAs (Koper *et al.*, 1980).

To confirm the adaptation of our established IgY-IgG ELISA system as an SEA detection method, SEA-containing culture samples from the food poisoning strains 196E and 11689 were used for the detection of SEA.

IMMUNOLOGY & MEDICAL MICROBIOLOGY

RESEARCH ARTICLE

Submucosal mast cells in the gastrointestinal tract are a target of staphylococcal enterotoxin type A

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Keywords
staphylococcal enterotoxin; emesis; mast cell.

Abstract

Staphylococcal enterotoxin A (SEA) is a leading causative toxin of staphylococcal food poisoning. However, it remains unclear how this toxin induces emesis in humans, primates, and certain experimental animals. To understand the mechanism of SEA-induced emesis, we investigated the behavior of SEA in the gastrointestinal (GI) tract *in vivo* using the house musk shrew (*Suncus murinus*). Immunofluorescence of GI sections showed that perorally administered SEA translocated from the lumen to the interior tissues of the GI tract and rapidly accumulated in certain submucosa cells. These SEA-binding cells in the submucosa were both tryptase- and FcεRIα-positive, suggesting these SEA-binding cells were mast cells. These SEA-binding mast cells were 5-hydroxytryptamine (5-HT)-positive, but the intensity of the 5-HT signal decreased over time compared to that of mast cells in the negative control. Furthermore, toluidine blue staining showed the number of metachromatic mast cells was decreased in the duodenal submucosa, suggesting that SEA binding induced degranulation and release of 5-HT from submucosal mast cells. These observations suggest that the target cells of SEA are submucosal mast cells in the GI tract and that 5-HT released from submucosal mast cells plays an important role in SEA-induced emesis.

Introduction

The primary symptoms of staphylococcal food poisoning are nausea and vomiting, which occur within 1–6 h after ingestion of the causative foods. In 1930, it was shown that this food poisoning was not caused from infection by *Staphylococcus aureus* but from intoxication by a heat-stable exotoxin (later termed an 'enterotoxin') produced by the bacterium in contaminated foods (Dack *et al.*, 1930). Over the past few decades, a number of studies have been conducted on the nature of enterotoxins and staphylococcal food poisoning. Today, staphylococcal enterotoxins (SEs) are recognized as the most common causative agents of human food poisoning worldwide (Kotb, 1995; Balaban & Rasooly, 2000). SEs are single-chain, heat-stable proteins that are 23–27 kDa in size and are resistant to proteolytic enzymes (Balaban & Rasooly, 2000; Li *et al.*, 2011). In the past, five serological types of classical SEs (SEA to SEE) had been recognized; however, the existence of 16 new types of SEs and SE-like (SEl) toxins (SEG to SElV) has been revealed in recent years (McCormick *et al.*, 2001; Omoe *et al.*, 2003; Thomas *et al.*, 2006; Ono *et al.*, 2008). In addition to their emetic activity, SEs and SEls are known as superantigens that can activate a large number of T cells in a selective manner through the direct association of Vβ elements in the β chain of the T cell receptor with major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (Uchiyama *et al.*, 2006). Moreover, certain SEs have been implicated as causative agents of toxic shock syndrome, in addition to toxic shock syndrome toxin 1 (TSST-1) (McCormick *et al.*, 2001). The

superantigenic properties of SEs have been intensively studied to understand the etiology of toxic shock syndrome. However, since Dack *et al.* suggested more than 80 years ago that SEs are the causative agents of staphylococcal food poisoning, the exact molecular and cellular mechanisms of the emetic activity of SEs still remain unclear.

This lack of progress in determining the mechanism of the emetic activity of SEs can be attributed to the lack of a suitable animal model for studying emesis. Historically, the primate model has been considered the gold standard for detecting the emetic activity of SEs and has been used to a limited extent to investigate the mechanism by which SE induces emesis (Bergdoll, 1988). This model has been used to demonstrate that the site of action for the induction of the emetic reaction is the abdominal viscera and that the SE ultimately stimulates the vomiting center of the medulla oblongata through the vagus and sympathetic nerves (Sugiyama & Hayama, 1965). However, the high cost and limited availability of primates have lead to a reduction in the investigation of SE-induced emesis.

On the other hand, the house musk shrew, *Suncus murinus*, has been recognized as an animal that responds to various emetic drugs. We have previously shown that the house musk shrew responds to many kinds of SEs, including SEA by peroral (PO) and intraperitoneal (IP) administration (Hu *et al.*, 1999, 2003a). Hence, the house musk shrew is a suitable animal model for investigating the mechanism of SE-induced emesis. Using this animal model system, it has been shown that SEA induces emesis through increase in the release of 5-hydroxytryptamine (5-HT) in the duodenum and that emesis is inhibited by 5-HT_3 receptor antagonists (Hu *et al.*, 2007). Based on these results, we had hypothesized that PO-administered SEA binds to certain 5-HT-positive target cells and induces the release of 5-HT from these cells in the gastrointestinal (GI) tract, with 5-HT subsequently stimulating the vomiting center via the afferent vagal nerves. A number of cell types in the GI tract contain 5-HT, including enterochromaffin (EC) cells, neuronal cells, and mast cells. Especially, it has been reported that intradermally injection of SEB caused immediate-type skin reaction through degranulation of cutaneous mast cells in cynomolgus monkeys (Scheuber *et al.*, 1985). Also, SEB induced release of 5-HT from murine mast cells *in vitro* (Komisar *et al.*, 1992). These facts lead us to a hypothesis that mast cells in GI tract may play a certain role in SEA-induced emesis. In this study, we attempted to clarify how SEA migrates in the GI tract tissue *in vivo* and to identify the cells that release 5-HT following SEA stimulation using a house musk shrew model. Here we demonstrate that the submucosal mast cells in the GI tract are a target of SEA and play an important role in SEA-induced emesis through degranulation and release of 5-HT.

Materials and methods

Animals and toxins

House musk shrews (*S. murinus*; Jic:Sn-Her, Nihon Clea, Tokyo, Japan) were used for the experiments after obtaining approval of the Animal Ethics Committee at Iwate University. The shrews were housed under controlled conditions of illumination (12/12 h light/dark cycle starting at 7 AM). The study was approved by the Iwate University Laboratory Animal Care Committee (Accession No. 201064). SEA and TSST-1 were expressed in a GST-fusion system. The preparation of recombinant toxins was performed as previously described (Hu *et al.*, 2003a, b).

Tissue preparation for immunostaining

House musk shrews were perorally administered 100 μg of SEA per animal [100 μg mL^{-1} in phosphate-buffered saline (PBS)] and euthanized by cervical dislocation at 15, 30, 60, and 90 min after SEA administration without anesthesia. Specimens of the GI tract were removed, washed with ice-cold PBS, and fixed overnight with Mildform 10NM (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Afterward, the tissue samples were soaked in 30% sucrose in PBS (pH 7.4) overnight at 4 °C and frozen in OCT medium (Sakura Finetech, Tokyo, Japan) at −80 °C. The tissue samples were also embedded by paraffin for hematoxylin and eosin (H&E) staining. Paraffin-embedded tissue samples were cut into 5-μm-thick sections. The sections were deparaffinized and stained with H&E.

Immunofluorescence analysis

Cryostat sections (6–10 μm) were processed by an immunofluorescence method for single and double labeling. The sections were washed in PBS and incubated with 5% skim milk in PBS/0.05% Tween20 (PBST) for 30 min at room temperature. Afterward, the sections were rinsed in PBST and incubated with primary antibodies. Rabbit polyclonal anti-SEA antibody (0.2 μg mL^{-1}), which had been prepared by immunizing rabbits with SEA (Shinagawa *et al.*, 1974), was purified by affinity chromatography using a HiTrap kit (GE Healthcare UK Ltd., UK), as previously reported (Omoe *et al.*, 2002). Rabbit polyclonal anti-TSST-1 antibody (0.1 μg mL^{-1}), which had been prepared by immunizing rabbits with TSST-1, was purified by precipitation with a saturated ammonium sulfate solution following affinity chromatography using a HiTrap kit, as previously described (Hu *et al.*, 2003b). Rat monoclonal anti-5-HT antibody (1 : 200; Millipore,

Billerica, MA), mouse monoclonal anti-tryptase antibody (clone AA1 1 : 1000; Dako Japan, Tokyo, Japan), mouse monoclonal anti-rat MHC class II RT1B antibody (clone OX-6 1 : 1000; BD Pharmingen, Franklin Lakes, NJ), and mouse monoclonal anti-FcεRIα antibody (clone CRA1 1 : 1000; BioAcademia, Osaka, Japan) were also used as primary antibodies. Incubation of sections with primary antibodies was carried out at 4 °C overnight. After incubation, the sections were rinsed in PBST and incubated with the following secondary antibodies: Alexa 488-conjugated goat anti-rabbit IgG, Alexa 568-conjugated goat antirat IgG, and Alexa 568-conjugated goat anti-mouse IgG (1 : 1000; Life Technologies Japan Ltd., Tokyo, Japan) for 60 min at room temperature. All of the above-mentioned antibodies were diluted in Can Get Signal Immunostain Solution A (TOYOBO Co., Ltd., Osaka, Japan). The sections were coverslipped with Prolong Gold antifade reagent (Life Technologies Japan Ltd.) and examined using a fluorescence microscope (AXIO Observer A1; Carl Zeiss Microimaging Co., Ltd., Tokyo, Japan).

Toluidine blue staining

For toluidine blue staining, specimens of the GI tract were removed from house musk shrews at 30 and 90 min after peroral administration of SEA. The specimens were fixed overnight with Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid). Afterward, the tissue samples were paraffin-embedded using standard procedures. Paraffin-embedded tissue samples were cut into 5-μm-thick sections. The sections were deparaffinized and stained with 0.1% toluidine blue solution.

Statistical analysis

Observation and quantification of toluidine blue-stained sections were carried out independently by two investigators. The numbers of metachromatic cells in the GI tract are presented as the mean ± SD of n observations per square millimeter. The statistical study was performed using ANOVA followed by the Tukey test. A P value of < 0.05 was considered statistically significant.

Results

SEA translocates from the lumen to the submucosa of the GI tract

For revealing the behavior of SEA in the GI tract of the house musk shrew after PO administration, an immunofluorescence technique was employed. The stomach and duodenum were collected at 15, 30, 60, and 90 min following SEA administration and then processed for immunofluorescence using an anti-SEA polyclonal antibody. Figure 1 shows sections of the mucosa and the submucosa of the duodenum and sections of the submucosa of the stomach. Non-SEA-administrate house musk shrews were stained by anti-SEA primary antibody as negative controls (Fig. 1a, f, k, and p). Figure 1u and v show H&E staining sections of the house musk shrew duodenum. After 15 min of administration, a few SEA-immunopositive and morphologically circular or elliptical form cells appeared in the submucosa of the stomach and duodenum (Fig. 1g, l, and q). There was no evident SEA signal in the mucosa of the duodenum, suggesting that SEA rapidly translocates from the lumen into the submucosa of the GI tract (Fig. 1b). Moreover, SEA was not observed to bind to EC cells in the luminal epithelium of the duodenum, indicating that EC cells may not play a role in SEA-induced emesis.

At 30–90 min after administration, there was a marked increase in the number of SEA-immunopositive cells in the GI submucosa (Fig. 1h–j). Moreover, in contrast to the circular or elliptical morphology of the SEA-positive cells at 15 min after administration (Fig. 1g), the morphology of SEA-positive cells from 30 to 90 min after administration of SEA was spindle shaped (Fig. 1m–o and r–t). On the other hand, few SEA-immunopositive cells were detected in the mucosa from 30 to 90 min after administration, although the intensity of signals observed in the lamina propria was markedly weak compared to that of signals in the submucosa (Fig. 1c–e). Specificity of signals obtained by anti-SEA antibody was confirmed by another negative control experiments by staining of sections from SEA-administrated house musk shrews and non-SEA immunized rabbit IgG as primary antibody. The non-SEA immunized rabbit IgG did not exhibit any significant signal on SEA-administrated house musk shrew sections (data not shown). These results suggest that SEA translocates across the mucosal epithelium, migrates into the submucosa, and binds target cells in the GI tract. However, the entrance site of SEA and the route of migration in the GI tract are still unknown.

SEA accumulates in submucosal mast cells in the GI tract

The morphology of the SEA-binding cells in the submucosa at 30–90 min after SEA administration did not correspond to the morphology of myenteric nerves. Therefore, we suspected that the target cells of SEA were the mast cells because SEB elucidates degranulation of cutaneous mast cells in monkeys (Scheuber *et al.*, 1985), and SEB also binds and induces 5-HT release from cultured rodent mast cells (Komisar *et al.*, 1992). For determining whether SEA binds to mast cells in the

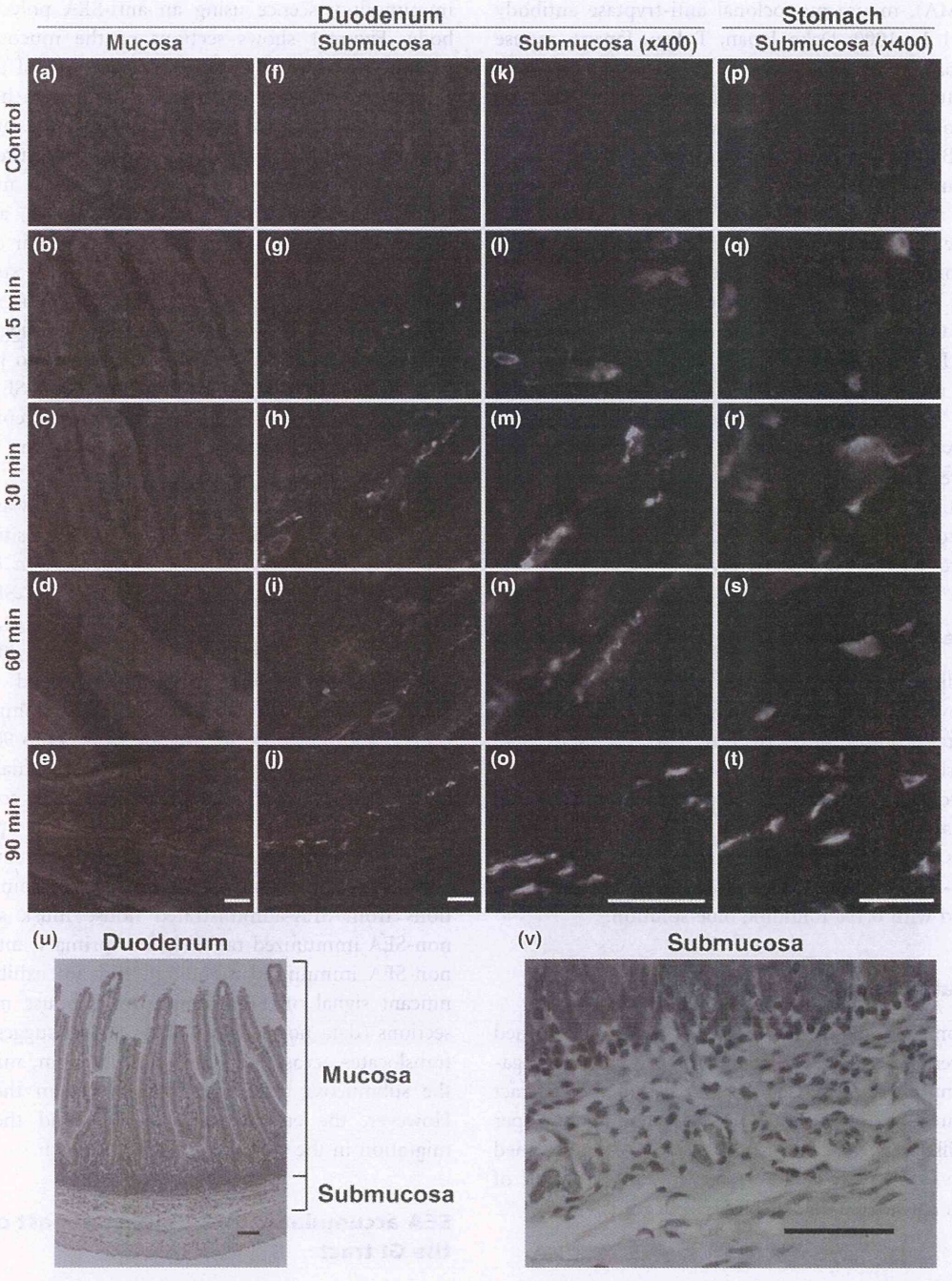

Fig. 1. SEA migrates from the lumen to the submucosa of the GI tract. The stomach and duodenum were collected at 15, 30, 60, and 90 min after administration of SEA and then processed for immunofluorescence using an anti-SEA polyclonal antibody. (a–e) Duodenal mucosa (×200). (f–j) Duodenal submucosa (×200). (k–o) Duodenal submucosa at a high-power magnification (×400). (p–t) Stomach submucosa (×400). The duodenal mucosa showed little or no signal following PO-administered SEA (b–e). However, a few SEA-binding cells appeared in the submucosa at 15 min after PO administration. These cells were circular or elliptical in form (g, l, and q). From 30 to 90 min after administration, the number and immunofluorescence intensity of the SEA-binding cells were markedly increased in the GI submucosa. These cells were spindle shaped (h–j, m–o, and r–t). Control experiments were performed using sections from non-SEA-administrate house musk shrews and anti-SEA primary antibody (a, f, k, p). Each scale bar is equal to 40 μm. H&E staining sections displayed the intestinal mucosa and submucosa of house musk shrew. (u) Duodenum stained by H&E. Scale bar is equal to 80 μm. (v) Corresponding to H&E stained duodenal submucosa at higher magnification. Scale bar is equal to 40 μm.

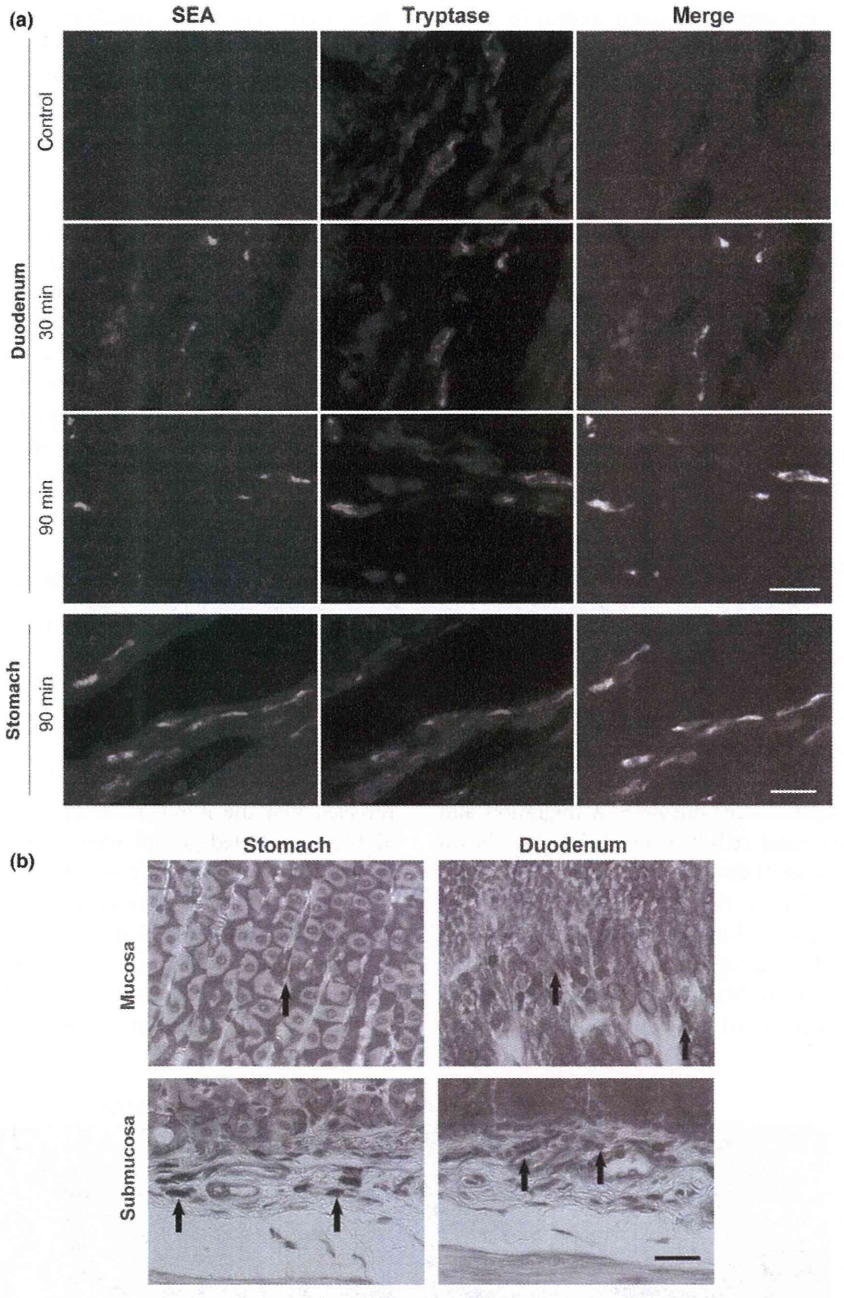

Fig. 2. SEA accumulates in submucosal mast cells in the GI tract. (a) GI sections were processed by double-staining immunofluorescence using anti-SEA antibody (green) and anti-tryptase monoclonal antibody (red), which recognizes the mast cell marker tryptase. From 30 to 90 min after administration, SEA bounds to the submucosal tryptase-positive cells in both the duodenum and stomach. Non-SEA-administrated house musk shrew sections were stained by anti-SEA antibody and antitryptase antibody as a control. (b) Toluidine blue staining revealed that metachromatic cells exist in both of the mucosa and the submucosa in the duodenum of house musk shrew. Arrows indicate representative metachromatic cells. Each scale bar is equal to 20 μm.

submucosa, the sections were processed by double-staining immunofluorescence using an anti-SEA antibody and an anti-tryptase monoclonal antibody, which recognizes the mast cell marker tryptase. As expected, the SEA and tryptase signals were co-localized in the sections 30–90 min after administration, indicating that the SEA-binding cells were mast cells (Fig. 2a). Mast cells were readily distinguishable from other type of cells by their

metachromatic staining properties when stained by toluidine blue. Metachromatic mast cells were present in both of the mucosa and submucosa in the GI tract as shown in Fig. 2b. However, SEA/tryptase double-positive cells were observed in the submucosa but not in the mucosa. To confirm these results, the sections were stained by an anti-FcεRIα antibody, which detects FcεRIα, the IgE receptor α subunit, and another mast cell maker. SEA/FcεRIα double staining revealed that almost all of the SEA-binding cells were FcεRIα-immunopositive (Fig. 3). In addition, similar results were obtained in the stomach, indicating that the target cells of SEA in the GI tract were most likely submucosal mast cells.

Comparison of behavior of SEA and nonemetic superantigen TSST-1 in GI tract of the house musk shrew

In contrast to cells in the sections obtained 30–90 min after administration of SEA, the SEA-positive circular cells observed in sections obtained at 15 min after SEA administration were tryptase-negative (Fig. 4a). This suggests that SEA binds to another cell type in the early stage of SEA translocation into GI tract tissues, and then SEA binds tryptase- and FcεRIα-positive submucosal mast cells at 30–90 min after administration. At present, the roles of these tryptase-negative cells during SEA migration and accumulation in the mast cells remain unclear. As shown Fig. 4b, SEA/MHC class II double staining at 90 min after administration has shown that those signals were not co-localized, suggesting submucosal mast cells do not express MHC class II molecules, and SEA binds unidentified receptor other than MHC class II. On the other hand, TSST-1 is a member of the staphylococcal superantigen toxins and shows significant structural similarity to SEA, but this toxin does not exhibit emetic activity. For determining whether SEA accumulation in mast cells of the GI tract was enterotoxin-specific, the behavior of PO-administered TSST-1 was observed. Immunostaining of GI sections obtained from tissues 90 min after TSST-1 administration showed that TSST-1 crossed the mucosal epithelium and attached to MHC class II-immunopositive cells in the submucosa (Fig. 4c), in contrast to SEA. Moreover, the TSST-1 signal did not co-localize to that for tryptase (Fig. 4d), suggesting SEA-specific binding to mast cells plays an important role in SEA inducing emesis. Specificity of signals obtained by anti-TSST-1 antibody was confirmed by staining of sections from TSST-1-administrated house musk shrews and non-TSST-1 immunized rabbit IgG as primary antibody (data not shown).

SEA induces degranulation in mast cells

To estimate the degranulation of the submucosal mast cell quantitatively, we compared the number of metachromatic staining cells in toluidine blue-stained duodenum sections obtained from SEA-nonadministrated control, 30 min after SEA administration, and 90 min after SEA administration (Fig. 5a and b). Toluidine blue staining revealed that the numbers of metachromatic cells in the SEA-administrated groups were significantly decreased in the duodenal submucosa compared to that of control. On the other hand, there were no differences in the numbers of metachromatic cells in the mucosa of the control and SEA-administrated shrews (Fig. 5b), suggesting submucosal mast cell degranulation after SEA administration. In addition, double immunostaining using anti-SEA

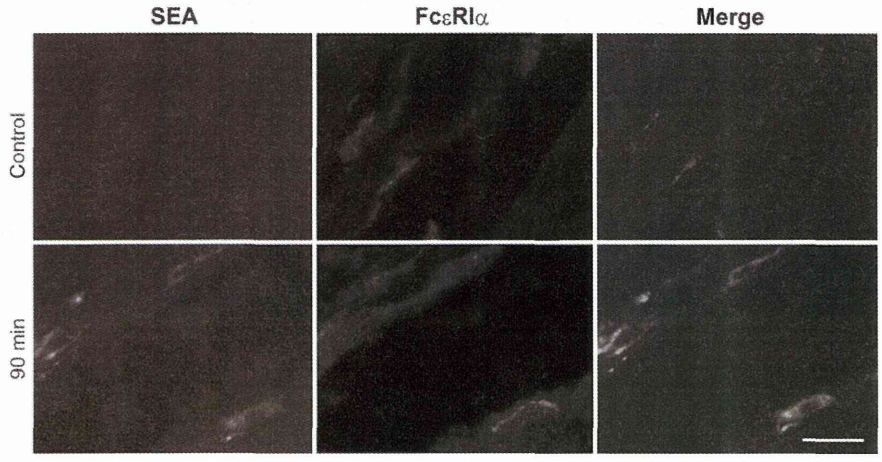

Fig. 3. GI sections were stained using anti-FcεRIα antibody (red), which detects the mast cell marker FcεRIα. SEA-immunopositive cells in the submucosa expressed FcεRIα at 90 min after PO administration.

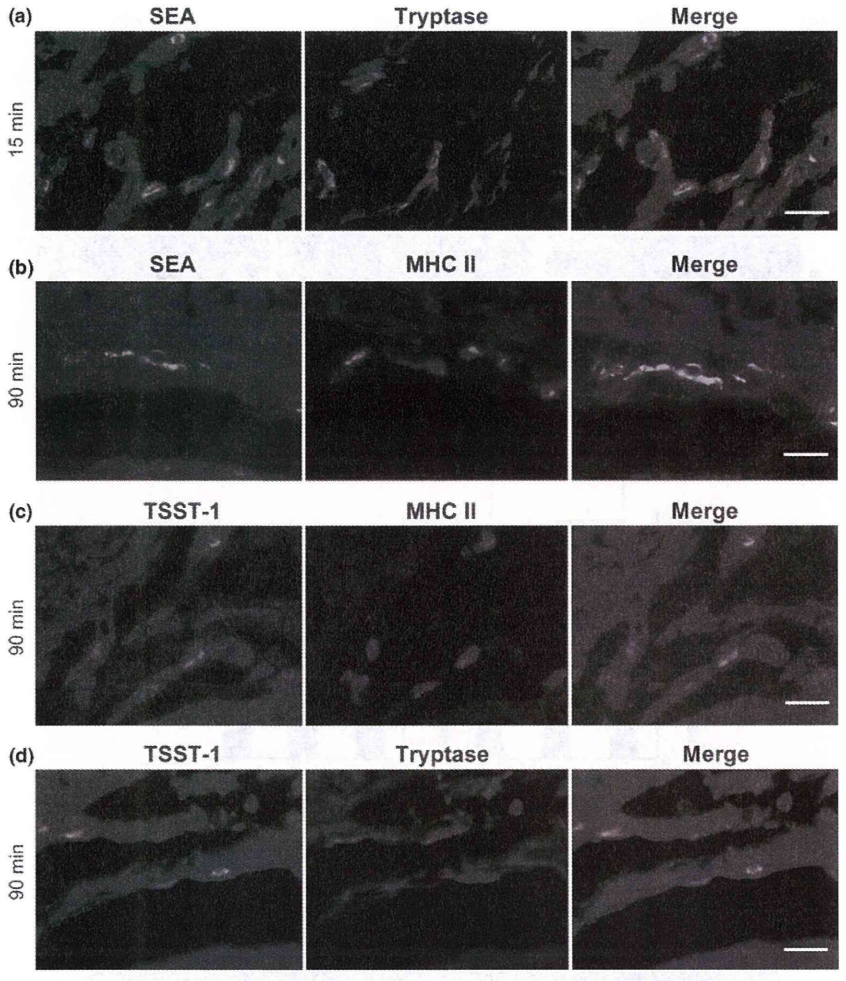

Fig. 4. SEA binds to tryptase-negative cells at an early time point, whereas TSST-1 binds to MHC class II molecule-positive cells at all time points. (a) SEA was not observed to co-localize with tryptase in the submucosal mast cells 15 min after PO administration. (b) SEA-binding cells did not exhibit MHC class II molecule in the section of 90 min after SEA administration. (c) TSST-1, a member of the staphylococcal superantigen toxins, crossed the mucosal epithelium and attached to MHC class II-immunopositive cells in the submucosa. (d) In contrast to SEA, the TSST-1 signal did not co-localize with the signal for tryptase at 90 min after TSST-1 administration. Each scale bar is equal to 20 μm.

antibody and anti-5-HT antibody showed that the SEA-immunopositive cells in the submucosa contained 5-HT, although the fluorescence intensity of the 5-HT signal decreased gradually from 30 to 90 min following SEA administration relative to that of the negative control (Fig. 5c). The decreasing 5-HT signal may reflect the degranulation of mast cells caused by the binding of SEA. Taken together, these results strongly suggest that SEA induces degranulation in submucosal mast cells.

Discussion

The main focus of the present study was to clarify how SEA migrates in the GI tract tissue *in vivo* and to identify the cells that release 5-HT following SEA stimulation using a house musk shrew model. Our present data showed that SEA translocates across the mucosal epithelium, migrates into the submucosa, and binds 5-HT-contained submucosal mast cells. Hu *et al.* (2007) have shown that SEA-induced emesis in the house musk shrew is inhibited by the 5-HT synthesis inhibitor p-chlorophenylalanine and the 5-HT_3 receptor antagonist granisetron, showing the important role of 5-HT in SEA-induced emesis in the house musk shrew. Moreover, 5-HT plays a significant role in the early phase of anticancer drug-induced emesis (Minami *et al.*, 2003). These anticancer drugs evoke 5-HT release from the EC cells, and 5-HT then stimulates the 5-HT receptor on the adjacent vagal

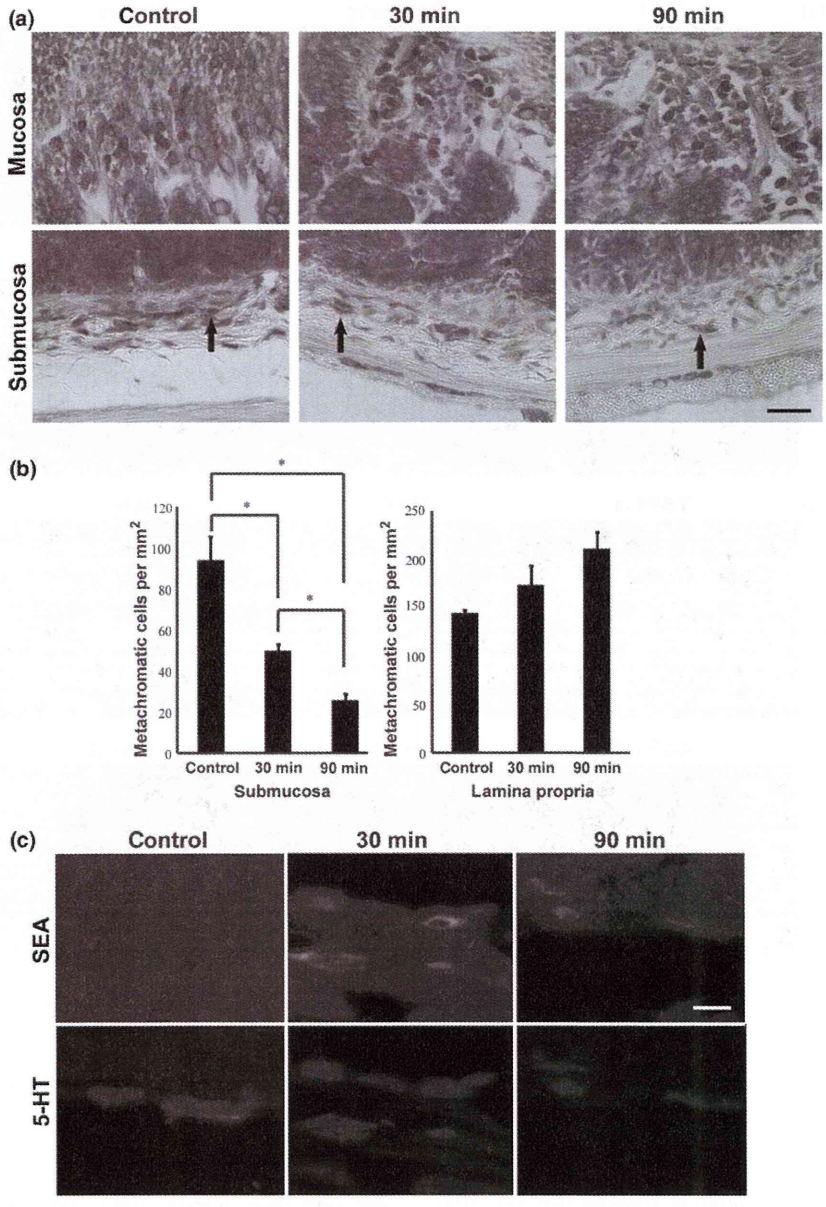

Fig. 5. SEA induces degranulation in submucosal mast cells. (a) The number of metachromatic cells in the submucosa was decreased in the section of 30 min and 90 min after administration of SEA, although there was no difference in the number of metachromatic cells in the mucosa. Arrows indicate representative metachromatic cells. Each scale bar is equal to 20 μm. (b) The effects of SEA on mucosal and submucosal mast cells in the duodenum. The administration of SEA markedly decreased the number of metachromatic cells in only the submucosa. The numbers of metachromatic cells are presented as the mean ± SD of *n* observations per square millimeter. Three sections were subjected to metachromatic cell counting at each time point. The statistical analysis was performed using ANOVA followed by the Tukey test. $^*P < 0.05$. (c) As shown by SEA/5-HT double immunostaining, the SEA-binding cells were 5-HT-immunopositive. However, the fluorescence intensity for 5-HT gradually decreased from 30 to 90 min after administration relative to that of the negative control staining non-SEA-administrated house musk shrew sections with anti-SEA antibody and anti-5-HT antibody. Scale bar is equal to 10 μm.

afferent nerves in the intestine. The resulting depolarization of the vagal afferent nerves stimulates the vomiting center in the medulla oblongata and eventually induces a vomiting reflex (Darmani & Ray, 2009). As shown in this study, submucosal mast cells in the GI tract play an important role in the release of 5-HT in SEA-induced emesis. In addition, it has been demonstrated that the emetic response to SEA is blocked by surgical vagotomy

in house musk shrews and primates (Sugiyama & Hayama, 1965; Hu *et al.*, 2007), suggesting that 5-HT released from submucosal mast cells may bind to the 5-HT_3 receptor expressed on enteric nerves in the GI tract and thereby induce the depolarization of these nerves.

Figure 6 shows a hypothetical mechanism for SEA-induced emesis in the house musk shrew. In this study, SEA crossed the mucosal epithelium in the GI lumen by an unknown mechanism and then accumulated in the submucosa. This translocation from the lumen to the submucosa occurred in 30–90 min, a timeframe that is consistent with the latency time of SEA-induced emesis in the house musk shrew (30–120 min) (Hu *et al.*, 1999, 2003a). In the stomach and duodenum of the house musk shrew, SEA finally binds to the tryptase- and FcεRIα-positive submucosal mast cells. The binding of SEA to an unidentified receptor expressed on the surface of submucosal mast cells induces their degranulation, resulting in the release of 5-HT. At present, it is unclear what type of molecule acts as an SEA receptor on the surface of submucosal mast cells. Superantigens including SEA bind to MHC class II molecules expressed on surface of antigen-presenting cells. It has been reported that MHC class II expression is not detected steady-state mouse bone marrow-derived mast cells, but MHC class II expression induced by Notch-1 mediated signaling through activation of transcription factor PU. 1 and class II transactivator promoter III (Nakano *et al.*, 2011). As shown in Fig. 4b, SEA and MHC class II signals were not co-localized in the sections 90 min after SEA administration, indicating receptor on mast cells is not MHC class II. Moreover, we suppose that unidentified SEA receptor on mast cells is capable to bind SEA more efficiently than MHC class II, so that orally administrated SEA shows tendency to bind mast cells rather than MHC class II-positive cells in GI tract. Further studies on the identification and molecular cloning of the unidentified SEA receptor gene are needed to understand the exact molecular basis of SEA-induced emesis and to elucidate the intracellular signal transduction pathway of the SEA receptor.

As shown in Fig. 4, nonemetic superantigenic toxin TSST-1 binds to MHC class II-positive cells but not mast cells. Recent study has shown that pepsin- and trypsin-digested TSST-1 fragment still maintains superantigenic and lethal shock activities (Li *et al.*, 2011). Schlievert *et al.* (2000) also reported that TSST-1 induced lethal shock via oral administration in endotoxin co-administrated rabbit model. These facts indicate that orally administered TSST-1 should be able to act as superantigen in GI tract, although TSST-1 is not capable to induce emetic reaction. This indicates that superantigenic activity of orally administrated TSST-1 does not implicate emetic

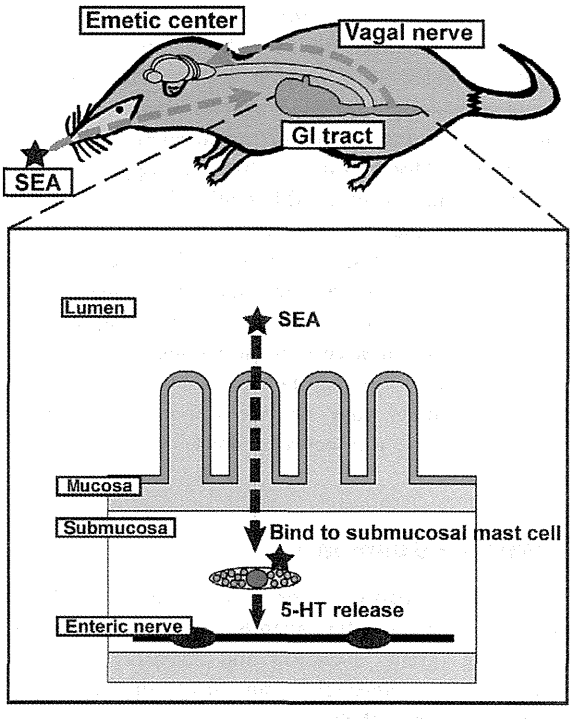

Fig. 6. The hypothetical mechanism of SEA-induced emesis in the house musk shrew. SEA administered by the PO route migrates from the GI lumen to submucosa and binds to target submucosal mast cells. The binding of SEA to an unidentified receptor on the surface of the submucosal mast cells then induces 5-HT release from the cells. Subsequently, the released 5-HT binds to the 5-HT_3 receptor expressed on enteric nerves and induces their depolarization. Finally, the depolarization of the vagal afferent nerves stimulates the vomiting center and induces the vomiting reflex.

reaction and activation of mast cells. On the other hand, SEA administered orally binds mast cells rather than MHC class II-positive cells, suggesting SEA dose not act as superantigen in this situation. These results suggest that superantigenic activity of toxins does not play an important role in inducing emesis when toxins are administrated orally.

A few previous studies have shown that SEB binds cultured mast cells and induces the release of mediators such as 5-HT (Komisar *et al.*, 1992). It has been assumed that only murine mast cells are capable of producing 5-HT; however, Kushnir-Sukhov *et al.* (2007) recently reported that human mast cells are also capable of synthesizing and releasing 5-HT. Based on these results and the ones from our study, we hypothesize that SEA also induces degranulation in submucosal mast cells in humans after ingestion of this toxin. As shown Fig. 2b, metachromatic mast cells were found in both the mucosa and submucosa in the GI tract. It is surprising that SEA binds only submucosal mast cells but not mast cells in the lamia

propria. It has been reported that mast cells exhibit phenotypic heterogeneity in different tissues and play significant roles in a number of important biological events such as IgE-mediated hypersensitivity, innate and adaptive immune response, angiogenesis, and tissue remodeling (Moon *et al.*, 2010). We suppose that the submucosal mast cells are differentiated to act as a target of SEA and play an important role in SEA-induced emesis. The present study represents the first observation of submucosal mast cell activation by orally administrated SEA using an *in vivo* system, suggesting important implications for mast cells in the pathogenesis of staphylococcal food poisoning. Further study of comparative mast cell biology may provide important information for bridging the gap between house musk shrews and humans.

Acknowledgements

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NOTE *Public Health*

Sensitivity of Hep G2 Cells to *Bacillus cereus* Emetic Toxin

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ABSTRACT. We herein examined the sensitivity of Hep G2 human hepatoma cells to *Bacillus cereus* emetic toxin. Hep G2 cells were treated with the emetic toxin, and the cell shape was observed. The same experiments were performed for comparison purposes, using HEp-2 cells, which are currently used by most laboratories for a bioassay of the emetic toxin. Hep G2 cells showed clearer vacuolation in the cytosol within 2 hr and required a shorter incubation period than HEp-2 cells (10 hr). The number of vacuoles in the Hep G2 cells was greater, and the size of the vacuoles was larger than those observed in HEp-2 cells. The minimal concentration of the emetic toxin required to induce the vacuolation of Hep G2 cells was 0.04 *ng*/m*l*. The concentration for the HEp-2 cells was 1 *ng*/m*l*. These findings indicate that Hep G2 cells show higher sensitivity to the emetic toxin. Hep G2 cells may be superior to the currently used HEp-2 cells for the bioassay of the emetic toxin.

KEY WORDS: *Bacillus cereus*, emetic toxin, hep G2 cell, vacuolation.

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Bacillus cereus food poisoning is classified into 2 types; one presents diarrheal, and the other emetic syndromes. Diarrheal syndrome in *B. cereus* food poisoning is caused by enterotoxin, and vomiting is triggered by an emetic toxin [16]. Several fatal cases of the emetic type of food poisoning have been reported in Switzerland (1997, [11]), Japan (2009, [17]), and Belgium (2011, [13]). The emetic toxin is a dodecapepsipeptide, with a molecular weight of 1153 [1]. The emetic toxin is a circlular peptide and is heat stable, i.e. resistant to cooking.

HEp-2 is a human cell line derived from a larynx carcinoma [12]. Hughes *et al.* reported that the emetic toxin caused vacuolation in the cytosol of HEp-2 cells [8]. Agata *et al.* isolated and purified the emetic toxin, using the vacuolation phenomenon in the HEp-2 cells as a marker indicating the active toxic fractions [1]. Because of the ease of cell culture, the use of the vacuolation assay employing HEp-2 cells has become wide-spread [2, 14, 18]. Although a color-developing assay monitoring the cell-death ratio has been proposed, non-specific cell-death was noted due to mitochondrial damages, when using this assay [6]. However, the HEp-2 cell assay also has many disadvantages. For instance, long-term personal experience is needed to recognize the vacuoles in the cytosol. Vacuolation does not occur in all of the cells treated, even those treated with higher concentration of the emetic toxin: only 10 to 30% of the cells show vacuolation. Long personal experience is needed to identify the vacuoles induced in HEp-2 cells. These characteristics of the HEp-2 cell assay suggest that the protocol requires improvement to be clearer, to provide more rapid results, and to be more convenient.

A direct cause of the fatal cases of *B. cereus* food poisoning was liver failure [11, 13, 17]. This finding indicated that the emetic toxin is hepato-toxic. The Hep G2 cell line is of human hepatoma origin [5]. We hypothesized that these liver cells would be useful for detecting the emetic toxin, and herein examined the sensitivity of Hep G2 cells to the emetic toxin.

The emetic toxin was purchased from Biocontrol Inc. (Nagoya, Japan), and the manufacturer indicated that the titer of the emetic toxin required to induce the vacuolation in HEp-2 cells was 1 *ng*/m*l*. The minimal effective concentration of the emetic toxin to induce vacuoles in HEp-2 cells was 0.5 to 2.0 *ng*/m*l* under our experimental conditions. The HEp-2 cells used in our study were kindly provided by Dr. Shigeko Ueda (Kagawa Nutrition University, Saitama, Japan). Hep G2 cells were purchased from the Human Science Foundation (Tokyo, Japan). HEp-2 cells were maintained in Basal Medium Eagle (BME, Sigma, St.Louis, MO, U.S.A.) containing 10% fetal bovine serum (FBS, Valley Biomedical, Winchester, VA, U.S.A.) and non-essential amino acids (Sigma). Hep G2 cells were maintained in Dulbecco's modified Eagle MEM (DMEM, Sigma) containing 10% FBS. Both cell lines were seeded into 25 -cm^2 flasks at 1 × 10^6 cells/flask, and then were incubated in a CO_2-incubator. The cells were used 2 days after seeding.

The bioassays using HEp-2 cells or Hep G2 cells were performed according to the previously reported method [1, 18]. Briefly, 25 μl of BME or DMEM was placed in every well of 96-well culture plates. A 1-μl aliquot of the emetic toxin (1 mg/m*l* in 75% methanol containing 250 mM KCl) was added into the first well containing 49 μl of the

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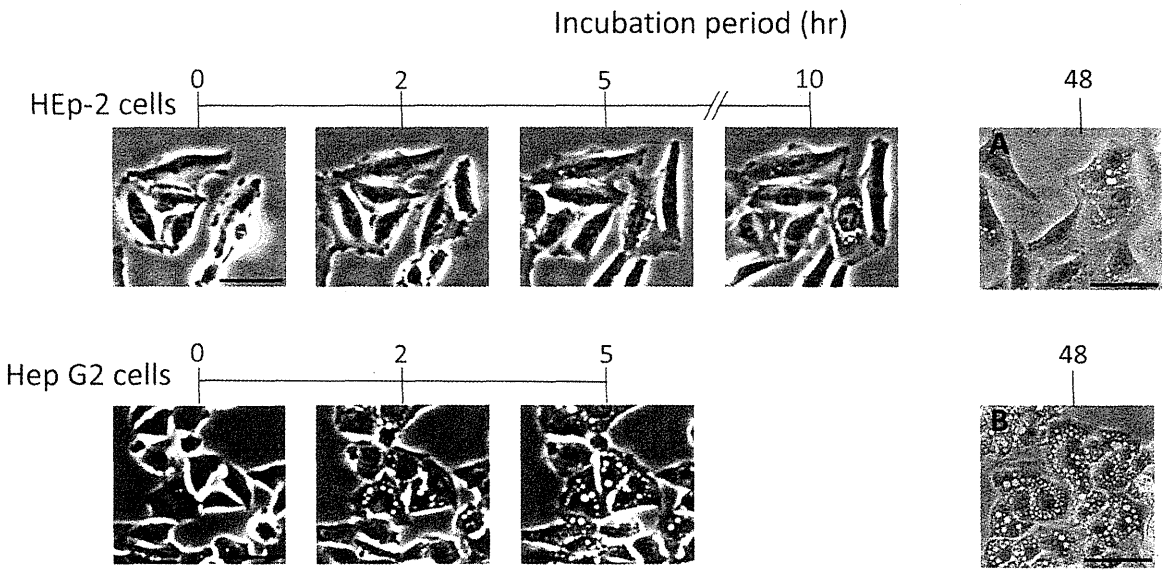

Fig. 1. The time course of the morphological changes of the HEp-2 and Hep G2 cells after treatment with *B. cereus* emetic toxin at 10 *ng*/m*l*. The suspended cells were seeded into 35-mm culture dishes, and incubated overnight. After *B. cereus* emetic toxin was added, the cells were recorded for changes in their shape every 15 min. Because the microscopic angle was fixed, individual cells could be consistently identified. The vacuolation caused by exposure to *B. cereus* emetic toxin (10 *ng*/m*l*) was independently recorded in HEp-2 (Panel A) and Hep G2 cells (Panel B) 48 hr after treatment. The bar shows 50 μm.

Table 1. Sensitivity of Hep G2 cells to *Bacillus cereus* emetic toxin

Day after Seeding	Cell conc.($\times 10^6$ cells/flask)		Minimal Dose of Toxin*(*ng*/m*l*)	
	Range	Median	Range	Median
1	1.0–1.3	1.1	2.4–9.8	4.4
2	1.9–2.2	2.0	0.01–0.08	0.04
3	3.1–4.1	3.8	0.6–2.4	1.4
4	5.3–6.5	5.8	2.0–4.9	3.0

Hep G2 cells were seeded into a 25-cm^2 flask at 1.0 x 10^6 cells/flask. Five flasks were used for each experiment day. * The minimal effective dose of *B. cereus* emetic toxin inducing vacuolation in Hep G2 cells.

medium, and the solutions were mixed. Thereafter, 25 μ*l* of the mixture was transferred to the neighboring well, mixed, and transferred repeatedly in the same way. The cells were harvested daily after seeding, the cellular concentration was counted, and then the cells were used for a bioassay to evaluate the sensitivity of the cells to the emetic toxin. Freshly suspended Hep G2 or HEp-2 cells (1×10^5 cells/m*l*) in BME or DMEM containing 1% FBS were added into wells at 100 μ*l*/well. The plates were then incubated in a CO_2 incubator at 37°C. The cells were photographed 24 or 48 hr after toxin treatment. More than 100 cells were counted and their vacuoles were identified on the photographs.

The HEp-2 and Hep G2 cells treated with the emetic toxin were observed under a Cell Observation Module (Model MCOK-F110-AS, Sanyo, Osaka, Japan), which has a fixed microscopic view. The module was placed in a CO_2 incubator, and was controlled with the software program provided by the manufacturer.

The emetic toxin was added into the 35-mm culture dishes containing attached HEp-2 and Hep G2 cells. Next, the shape of both cell lines was recorded every 15 min at a fixed angle. Small and unclear vacuoles appeared in the cytosol of HEp-2 cells 5 hr after toxin treatment. The vacuoles became clearer and typical after a 10-hr treatment period (Fig. 1). On the other hand, the shape of the Hep G2 cells changed more quickly. Clear and distinctive vacuoles were found only 2 hr after addition of the toxin. The vacuoles in the Hep G2 cells were larger, and the number of vacuoles was increased in comparison to those of the HEp-2 cell. Only 2 to 5% of the HEp-2 cells were vacuolated after 12 hr of treatment, while 25 to 60% of the Hep G2 cells were vacuolated after 12 hr. After a 24-hr incubation, the vacuolation rate of the HEp-2 cells was 20 to 30%, and that of the Hep G2 was 60% or more. The number of vacuole-bearing cells in the well containing Hep G2 cells was also higher than that in the HEp-2 cells 48 hr after toxin treatment (Fig. 1, panels A

and B). Therefore, at all time points examined, the number of vacuoles in the Hep G2 cells was higher, and the vacuoles were larger and more clearly distinguishable than those of HEp-2 cells.

The minimal concentration of the emetic toxin required to induce vacuoles in the HEp-2 cells prepared 2 days after seeding ranged from 0.5 to 2 *n*g/m*l*. The minimal effective concentration for Hep G2 cells was examined every day after the seeding of the cells. The concentrations were changed, depending on the cell number (Table 1). On day 2 after seeding, the cell concentration reached 2×10^6 cells/flask, and a small amount of the toxin was observed to induce vacuoles in the Hep G-2 cells: namely, a concentration range from 0.01 to 0.08 *n*g/m*l*, with a median of 0.04 *n*g/m*l*, was able to induce vacuoles formation. On days 3 and 4, the cells proliferated, and the minimal effective concentrations were found to be higher than those on day 2.

There are several methods that can be used to detect and quantify the concentration of emetic toxin, such as LC/MS [4, 7], bore sperm [3, 9, 15], isolated mitochondria [10], and cultured cells [2, 14, 18]. Each method has advantages and disadvantages; cost, required skill level of the technician, maintenance, difficulty to perform, time required for results, etc. For example, LC/MS instruments are expensive, rats have to be sacrificed to prepare isolated mitochondria, and bore sperms are generally unfamiliar to inspectors working in the laboratories examining food poisoning. Due to the ease of performing, and to the low cost, bioassays using cultured cells should be considered. The Hep G2 cells were found to show useful properties to detect and quantify the emetic toxin. The higher sensitivity of Hep G2 cells to the emetic toxin might contribute to the development of a new bioassay. Furthermore, because the Hep G2 cell line was established from human hepatoma, these cells might be useful to study liver failure resulting from exposure to the emetic toxin.

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Sensitivity of Hep G2 Cells to *Bacillus cereus* Emetic Toxin

Comments

[Q1] Medline reports the last page should be "7472" not "7422" in reference 4 "Biesta-Peters, Reji, Blaauw, In 't Velt, Rajkovic, Ehling-Schulz, Abee, 2010".

[Q2] Medline reports the first author should be "Finlay WJ" not "Finley WI" in reference 6 "Finley, Logan, Sutherland, 1999".

[Q3] CrossRef reports the last page should be "11" not "12" in reference 8 "Hughes, Bartholomew, Hardy, Kramer, 1988".

[Q4] Medline reports the first author should be "Jääskeläinen EL" not "Jääskelänien EL" in reference 9 "Jääskelänien, Teplova, Andersson, Andersson, Tammela, Andersson, Pirhonen, Saris, Vuorela, Salkinoja-Salonen, 2003".

[Q5] Medline reports the first author should be "Schoeni JL" not "Schoeni JJ" in reference 16 "Schoeni, Wong, 2005".

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＝調 査＝

レトルトパウチ詰コーンスープの原材料から分離されたフラットサワー菌の耐熱性と間欠滅菌法による制御の試み

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Thermal Resistance of Flat Sour Spoilage Bacteria Isolated from Food Ingredient of Retortable Pouched Corn Soup and the Control by Intermittent Sterilization

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緒 言

レトルト食品は缶詰に代わる簡便な保存食品として1950年代に研究開発が始まり，普及してきた．その国内生産量は年間約30万トンで，右肩上がりで増加しており今後も伸び続けると予想される．レトルト食品には「そのpHが4.6を超え，かつ，水分活性が0.94を超える容器包装詰加圧加熱殺菌食品にあっては，中心部の温度を120℃で4分間加熱する方法又はこれと同等以上の効力を有する方法であること」が食品衛生法で定められている．しかしながら，残存する耐熱性細菌芽胞が保管中に発芽増殖し，ガスを産生せずに酸を産生することで，酸味を呈するフラットサワー型変敗（以下フラットサワー）を引き起こすことをCameronらが報告した[1]．以後，アスパラガス缶詰や濃縮牛乳において発生の報告がある[3,4]．原因菌としては低酸性食品の場合は*Geobacillus stearothermophilus*が，酸性食品の場合は*Bacillus coagulans*が知られている[5]．すなわち，上記の加熱殺菌条件だけでは，食品衛生法の容器包装詰加圧加熱殺菌食品の製造基準の「原材料に由来して当該食品中に存在し，かつ，発育しうる微生物を死滅させるのに十分な効力を有する方法であること」を達成できない場合があることが判明している．

A食品会社が試験的に自社製レトルトパウチ詰コーンスープ製品（以下コーンスープ）を，夏季には室温が極めて高くなるトタン張り簡易建屋に半年以上保管していたところ，コーンスープは官能試験で酸味を呈し，フラットサワーが発生した．そこで，本研究ではフラットサワーが発生したコーンスープの原材料についてフラットサワー菌芽胞数の測定と分離菌の同定を試みた．また，分離菌株の芽胞の耐熱性を調べ，間欠滅菌法[3]を併用することにより，加圧加熱殺菌条件を厳しくすることなくフラットサワーのリスクを低減できるか否かについて，基礎的検討を行った．

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材料および方法

1. 供試材料

フラットサワーが発生したコーンスープ（容器外寸法：120×190 mm，pH 6.4）と同一ロットの原材料であるコーンペースト（ペースト状液体），てりやき粉（粉末）およびホワイトルウ（ペースト状）を用いた．

2. 使用培地および希釈液の調製

1) Dextrose Tryptone Agar（以下DTA）

Bacto Tryptone Pancreatic Digest of Casein（Becton Dickinson，以下BD）10 g，ブドウ糖（無水，和光純薬工業）5 g，ブロモクレゾールパープル（和光純薬工業）0.04 g，寒天粉末（和光純薬工業）12 gを純水1,000 m*l*に混合した後，オートクレーブで滅菌（121℃，15分，以下同じ）した．

2) Dextrose Tryptone Broth培地（以下DTB）

DTAと同組成で寒天を加えずに調製し，中試験管に10 m*l*ずつ分注しアルミキャップで栓をして，オートクレーブで滅菌した．

3) 0.3% 酵母エキス添加普通寒天培地（以下YNA）

Nutrient broth (OXOID) 10 g，酵母エキス (OXOID) 3 g，寒天粉末（和光純薬工業）20 gを純水980 m*l*に混合し，オートクレーブで滅菌した後，以下のA液およびB液を各10 m*l*ずつ加え，よく混合してから20 m*l*ずつシャーレに分注し，平板にした．

A液：塩化マンガン(II) 四水和物（和光純薬工業）2.5 g，硫酸マグネシウム七水和物（ナカライテスク）25 g，硫酸鉄(II) 七水和物（和光純薬工業）30 mgを0.01 N塩酸（和光純薬工業）1,000 m*l*に混合し，オートクレーブで滅菌した．

B液：塩化カルシウム二水和物（和光純薬工業）15 gを0.01 N塩酸（和光純薬工業）1,000 m*l*に混合し，オートクレーブで滅菌した．

4) リン酸緩衝生理食塩水（以下PBS）

リン酸二水素カリウム（和光純薬工業）34 gを純水500 m*l*に溶解後，1 N水酸化ナトリウム（和光純薬工業）溶液175 m*l*を加え，純水で全量を1,000 m*l*として，pH 7.2に調整した．これを原液として冷蔵保管し，使用時には原液1 m*l*を純水800 m*l*に混合し，塩化ナトリウム（和光純薬工業）を0.85% (w/v) となるように加えてオートクレーブで滅菌した．

3. 供試材料のフラットサワー菌芽胞数の測定

供試材料約10 gを滅菌ストマフィルター（Sタイプ；GSIクレオス）に採り，System Diluter (IUL, S.A Barcelona, Spain)でPBSを10倍希釈量加えた．これをマスティケーター (IUL) で90秒間よく混和させ，沸騰水中で30分間加熱処理し，試料液とした．試料液は以下の測定に供した．

1) 平板培養法

試料液2 m*l*ずつを滅菌シャーレ10枚に加えDTA 18 m*l*で混釈し，平板にした．これら平板10枚は5枚を37℃で，残りの5枚を55℃で3日間培養した．培養後，平板5枚当たりの黄色集落を計数し，供試材料1 gに含有するフラットサワー菌芽胞数とした．

2) MPN法

試料液10 m*l*, 1 m*l*, 0.1 m*l*を各3本のDTB に接種し，55℃で3日間培養した．ただし，10 m*l*を接種する場合には2倍濃縮のDTBを用いた．DTBが黄変した場合にはその1白金耳をDTAに画線し，55℃で3日間培養した．黄変した集落が形成された場合を陽性とした．希釈3段階の各3本のDTBの陽性本数から最確数表により供試材料1 g当たりのフラットサワー菌芽胞数を求めた．

4. 供試材料からのフラットサワー菌の分離と同定

フラットサワー菌が検出された供試材料のDTA平板から1菌株ずつ分離し，DTBで純培養した．分離菌株の菌種の同定はMicroSeq法[8]で行い，MicroSeq Microbial Identificationを使用して，供試菌株の16S rRNA遺伝子の5′末端側500 bpの塩基配列を決定した．得られたデータは，MicroSeqシステム (Applied Biosystems: MicroSeq® ID Analysis v2.0) でホモロジー検索を行った．

5. 供試材料から分離した菌株の芽胞形成と耐熱性

1) 供試菌株の芽胞懸濁液の作製

供試菌株をDTBに接種し，55℃で1日間培養した後，その培養液0.3 m*l*をYNAに添加し滅菌コンラージ棒で塗抹した．これらの平板は55℃で4日間培養した後，平板表面の菌苔を白金耳で回収しYNA 1枚分を0.5 m*l*のPBSに懸濁し，20分間煮沸して栄養細胞を死滅させ，芽胞懸濁液とした．芽胞懸濁液の10^6倍までの10倍段階希釈液をPBSで希釈して作製し，各段階希釈液を2枚の平板に1 m*l*ずつ分注し，それぞれをDTA 19 m*l*で混釈した．これら平板は55℃で3日間培養し，集落を30～300 CFU計数できた希釈段階における平板2枚の集落数平均値を生残芽胞数とした．この集落計数の方法を以下，混釈平板法と示す．芽胞懸濁液の初発芽胞数は，コーンペースト由来株が2.0×10^8 CFU/m*l*，てりやき粉由来株が1.1×10^8 CFU/m*l*であった．芽胞懸濁液は−45℃で凍結保存した．

2) 芽胞の耐熱性の測定

供試菌株の芽胞懸濁液をPBS 15 m*l*で10^6 CFU/m*l*になるよう希釈し，その1 m*l*ずつをTDTチューブ（内径4 mm，外径 7 mm，長さ100 mm；マルエム）に分注した．開口部は回転式自動アンプル溶封器（マルエム）を用いて溶封した．これらは恒温油槽（EYELA OHB-2000，シリコンオイル）を用いて，コーンペースト由来株については115℃で15, 30, 45, 60, 75, 90分間，118℃で10, 20, 30, 40, 50, 60分間および120℃で5, 10, 15, 20, 25, 30分間の加熱処理を行い，てりやき粉由来株については120℃で 1, 2, 3, 4, 5, 6分間の加熱処理を行った．加熱処理後のTDTチューブはガラス管切で切断し，混釈平

板法で生残芽胞数を求め，生残曲線を作成した．なお，加熱処理をしていないものを加熱時間0分のデータとした．生残曲線からD値を，加熱致死時間曲線からz値を求めた[7]．

6. 供試菌株の芽胞の発芽時間の検討

分離菌株の間欠煮沸による滅菌を検討するうえで，最も発芽率が高い培養時間を検討した．供試菌株の芽胞数が10^6 CFU/mlになるよう芽胞懸濁液をDTB 10 mlに添加し1 mlずつマイクロチューブ（greiner bio-one, 1.5 ml容）に分注して55℃で12, 18, 24時間培養した．所定の条件で培養したチューブは直ちに沸騰水中で20分間煮沸し栄養細胞を死滅させた後，混釈平板法で集落を計数し生残芽胞数を求めた．初発芽胞数と加熱後の生残芽胞数の差をもって発芽芽胞数とした．また，各培養条件のチューブは2本ずつ用意し，その平均値を結果に用いた．

7. 供試菌株の間欠煮沸による芽胞の滅菌法の検討

芽胞数が10^6 CFU/mlになるよう供試菌株の芽胞懸濁液をDTBおよびコーンスープ（フラットサワーが発生した製品と別ロットの製品）15 mlに添加し，1 mlずつをマイクロチューブに分注した．これらのチューブは発芽促進のため沸騰水中で20分間加熱処理して55℃で24時間培養し，さらに培養後沸騰水中で20分間加熱処理する間欠煮沸を3日間繰り返した．各加熱処理後の試料の生残芽胞数を，混釈平板法を用いて調べた．

結　果

1. 供試材料中のフラットサワー菌芽胞数測定

供試材料のフラットサワー菌芽胞数の測定結果をTable 1に示した．供試材料中のフラットサワー菌芽胞は55℃培養でのみ検出された．コーンペーストおよびてりやき粉中の芽胞数は，平板培養法ではそれぞれ11 CFU/g, 3 CFU/gで，MPN法ではいずれも2.3 MPN/gであった．ホワイトルウからフラットサワー菌芽胞は検出されなかった．

2. 供試材料から分離したフラットサワー菌の同定

フラットサワー菌が検出されたコーンペーストおよびてりやき粉のDTA平板から1株ずつを分離し，コーンペースト由来株をCP株，てりやき粉由来株をTK株とした．CP株およびTK株について，16S rRNA遺伝子解析で同定を行ったところ，CP株は相同性99.26%で*G. stearothermophilus*と，TK株は相同性97.32%で*G. thermoglucosidasius*と同定された．

Table 1. Number of flat sour bacteria spores found in corn paste, teriyaki powder and white roux by method of aerobic plate count and MPN

	Aerobic plate counts (CFU/g)		MPN method (MPN/g)
	37℃	55℃	55℃
Corn paste	0	11	2.3 (0.4-12)*1
Teriyaki powder	0	3	2.3 (0.4-12)*1
White roux	0	0	0

*1 95% confidence limit

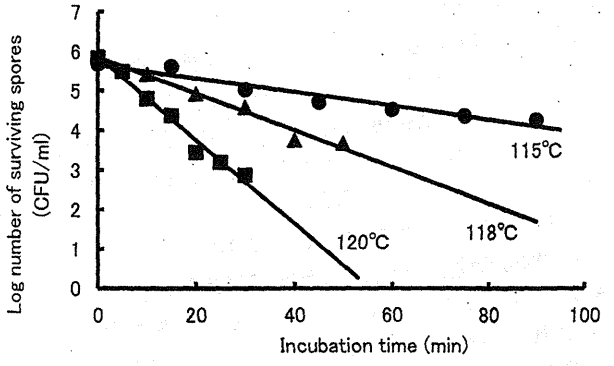

Fig. 1. Survival curve for bacterial spores of *G. stearothermophilus* of CP strain heated in PBS (pH 7.2).
Their heating temperatures and regression equations ware ●, 115℃, $y=-0.017x+5.655$ ($R^2=0.937$), ▲, 118℃, $y=-0.046x+5.860$ ($R^2=0.984$), ■, 120℃, $y=-0.105x+5.876$ ($R^2=0.980$).

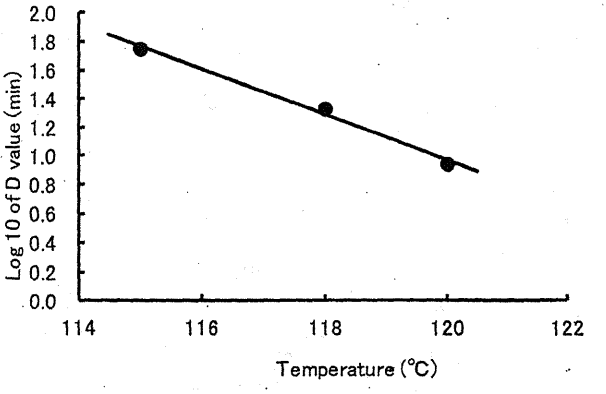

Fig. 2. The z value of spores of *G. stearothermophilus* of CP strain in PBS (pH 7.2).
The regression equation: $y=-0.159x+20.11$ ($R^2=0.992$)

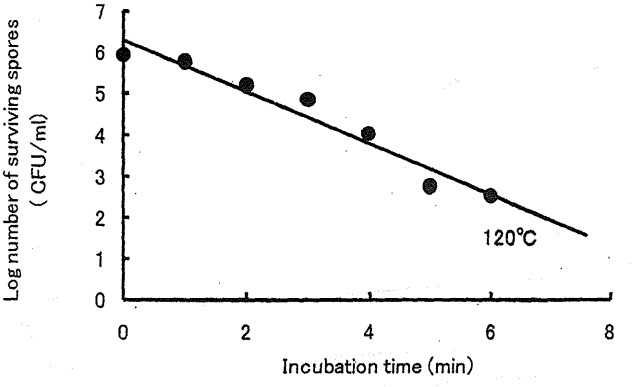

Fig. 3. Survival curve for bacterial spores of *G. thermoglucosidasius* of TK strain heated in PBS (pH 7.2) at 120℃.
The regression equation: $y=-0.619x+6.285$ ($R^2=0.949$).

3. 供試材料から分離した菌株の芽胞の耐熱性

CP株のPBS (pH 7.2)中における耐熱性試験結果をFig. 1およびFig. 2に，TK株のPBS (pH 7.2)中における120℃の生残曲線をFig. 3に示した．またこれらの試験結果をTable 2に示した．CP株のPBS (pH 7.2)中における$D_{115℃}$値は56.2分，$D_{118℃}$値は21.4分および$D_{120℃}$値は9.3分で，z値は6.3℃であった．TK株のPBS (pH 7.2)中における$D_{120℃}$値は1.6分であった．フラットサワーが発生した製品は製造工程において120℃で16分間の加熱殺菌が施されていたことから，$D_{120℃}$値が1.6分のTK株がフラットサワーを引き起こした可能性は低いと判断し，以後の実験はCP株に限定して実施した．

4. 供試菌株の芽胞の発芽に及ぼす培養時間の影響

CP株芽胞のDTB中における55℃培養での芽胞数を測定した結果をFig. 4に示した．煮沸によって減少する菌数を発芽数とした．CP株は55℃では12時間後までほとんど発芽せず生芽胞数は3.3×10^6 CFU/mlであったが，18時間および24時間後には8.0×10^5 CFU/mlになり，初発芽胞数の約76%が発芽した．

5. 供試菌株の芽胞の発芽に及ぼす間欠煮沸の影響

CP株芽胞を添加したDTBおよびコーンスープ中における間欠煮沸による生芽胞数の変化をFig. 5に示した．初発芽胞数はいずれの試料とも7.1×10^6 CFU/mlであったが，3回目の煮沸後にはDTB中では生芽胞数は1.3×10^5 CFU/mlと初発芽胞数より約98%減少した．一方，

Table 2. Thermal resistance of bacterial spores of *G. stearothermophilus* of CP strain and *G. thermoglucosidasius* of TK strain in PBS (pH 7.2)

Isolates	*D* value (min)			*z* (℃)
	115℃	118℃	120℃	
CP	56.2	21.4	9.3	6.3
TK	NT	NT	1.6	NT

NT: Not tested

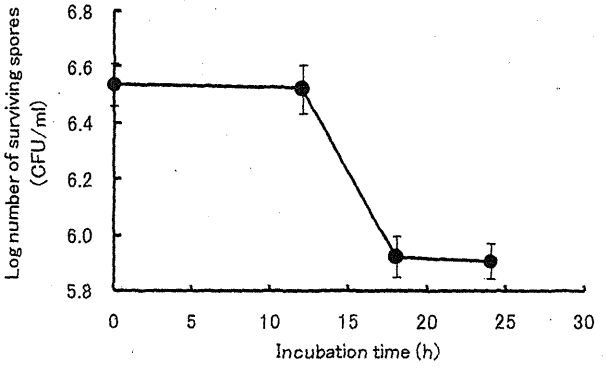

Fig. 4. Number of surviving bacterial spores of *G. stearothermophilus* of CP strain subjected to boiling after the incubation in Dextrose Tryptone Broth for 12, 18, and 24 h at 55℃. It took more than 18 h for the CP strain spores to germinate in the broth.

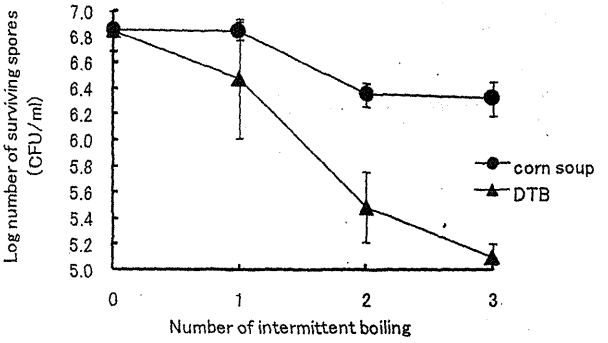

Fig. 5. Survival curve for bacterial spores subjected of *G. stearothermophilus* of CP strain under intermittent boiling at Dextrose Tryptone Broth and corn soup (pH 6.4).

コーンスープ中では2.1×10^6 CFU/mlまでの減少にとどまった．

考　察

フラットサワーが発生したレトルトパウチ詰コーンスープについて使用原材料を調べたところ，コーンペーストとてりやき粉から耐熱性芽胞を有する高温菌が検出された．16S rRNA遺伝子の塩基配列をもとに同定したところ，コーンペースト由来CP株は*G. stearothermophilus*，てりやき粉由来TK株は*G. thermoglucosidasius*と同定された．

今回フラットサワーが発見されたコーンスープは120℃で16分間の加熱工程を経ていた．てりやき粉由来TK株の120℃におけるD値が1.6分であったことから判断して，同株が当該コーンスープのフラットサワーに関与していた可能性は低い．また，コーンペースト由来CP株を別ロットのコーンスープに接種し，55℃で3日間培養したところ，コーンスープのpHが6.4から4.6に低下した（データは示さず）．以上のことから，コーンペースト由来CP株の*G. stearothermophilus*がフラットサワーの原因と考えられる．

A食品会社が試験的にコーンスープを保管していたトタン張り簡易建屋は，断熱性がある建築素材を使っておらず，夏季は50℃前後の高温になっていたと考えられる．そのため高温菌である*G. stearothermophilus*が発育可能な条件になったと推察される．実際には保管温度を40℃以下にすることで本菌によるフラットサワーを阻止できると考える．しかし，偶発的に保管温度が40℃以上になる可能性もあり，その場合のフラットサワーを防止するために間欠滅菌法の適用を試みた．

本製品において官能試験を合格できる最長の加熱殺菌条件は120℃で16分間以内であり，これ以上の加熱殺菌を実施することは難しい．そこで，間欠滅菌法が古くから実験器具などの芽胞菌の滅菌に使用されていた[2]ので，今回は同法を食品に応用し，中心温度120℃で4分間以上の加圧加熱殺菌と併用することによるフラットサワー