Fatal hemorrhage induced by Subtilase cytotoxin from Shiga-toxigenic *Escherichia coli*

Running title; SubAB-induced severe inflammatory hemorrhage in mice.

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Abstract

Subtilase cytotoxin (SubAB) is an AB₅ type toxin produced by a subset of Shiga-toxigenic *Escherichia coli*. The A subunit is a subtilase-like serine protease and cleaves an endoplasmic reticulum chaperone BiP. The B subunit binds to a receptor on the cell surface. Although SubAB is lethal for mice, the cause of death is not clear. In this study, we demonstrate in mice that SubAB induced small bowel hemorrhage and a coagulopathy characterized by thrombocytopenia, prolonged prothrombin time and activated partial thromboplastin time. SubAB also induced inflammatory changes in the small intestine as detected by ¹⁸F-fluoro-2-deoxy-D-glucose positron emission tomography imaging and histochemical analysis. Using RT-PCR and ELISA, SubAB was shown to increase interleukin-6 4. in a time-dependent manner. Thus, our results indicate that death in SubAB-treated mice may be associated with severe inflammatory

response and hemorrhage of the small intestine, accompanied by coagulopathy and IL6 production.

Key words; Subtilase cytotoxin; hemorrhage; coagulopathy; inflammation

1. Introduction

Infections with Shiga-toxigenic *Escherichia coli* (STEC) are characterized by gastrointestinal disease including hemorrhagic colitis, and may progress to systemic complications including hemolytic uremic syndrome (HUS) and cerebromeningitis. Toxins produced by STEC include Shiga toxins 1 and 2, and subtilase cytotoxin (SubAB). The latter toxin was recently discovered in O113:H21 strain 98NK2, which was responsible for an outbreak of HUS [1]. SubAB is now known to be produced by a variety of serotypes of STEC[2, 3]. However, O157:H7, the most common serogroup implicated in hemorrhagic colitis and HUS, does not produce SubAB [3]. SubAB contains one 35-kDa A subunit (SubA), and a B subunit (SubB) complex, which consists of a pentamer of 13-kDa monomers. The catalytic A subunit is a subtilase-like serine protease, and B subunits bind to receptors on target cells (2). Potential SubAB

receptors include *N*-glycosylated membrane proteins, $\alpha 2\beta 1$ integrin [4], and glycans terminating in the sialic acid *N*-glycolylneuraminic acid (Neu5Gc) [5, 6].

In Vero cells, SubAB treatment induced transient inhibition of protein synthesis, cell cycle arrest, and cell death [7]. These events were attributed to cleavage of the endoplasmic reticulum chaperone BiP by SubAB[8], which leads to ER stress secondary to accumulation of unfolded proteins. SubAB triggered the three major branches of the unfolded protein response (UPR) including the IRE1-XBP1, PERK, and ATF6 pathways [9]. SubAB induced phosphorylation of Akt and consequent activation of NF κ B through the ATF6 pathway [10]. Moreover, ER stress by SubAB down-regulated gap junction expression and inhibited functional gap junction intracellular communication[11]. Thus, SubAB-mediated ER stress by cleavage of major chaperone BiP triggers a number of abnormalities in cellular functions.

Wang et al. demonstrated that intraperitoneal administration of SubAB in mice induced microvascular thrombosis and other histopathological changes in the kidney, brain, spleen, and liver, and also induced significant neutrophil infiltration and apoptosis in liver, kidney, and spleen [12]. These findings indicated that not only Shiga toxin 1 or 2 but also SubAB may be involved in human STEC disease. However, tissue tropism of SubAB or mechanism by which SubAB induced organ damage *in vivo* has not been determined. To define the precise mechanism of disease pathogenesis, we identified main target organs of SubAB using histopathological, biochemical, and ¹⁸F-fluoro-2-deoxy-D-glucose positron emission tomography (¹⁸F-FDG PET) imaging analysis. In this report, we show that intraperitoneal administration of SubAB in mice induced severe inflammatory hemorrhage of the small intestine associated with increased interleukin-6 (IL6) mRNA and IL6 expression. A SubAB-associated

coagulopathy was characterized by thrombocytopenia, prolonged prothrombin time, and activated partial thromboplastin time. SubAB induced splenic atrophy, hyperplasia of the juxtaglomerular apparatus (JGA) and mesangial proliferation in kidney, but not apoptosis.

2. Results

2.1. Effect of SubAB on mice

To determine the toxicity of SubAB, mice were injected intraperitoneally with 10 µg of SubAB, mutant SubA(S272A,H89A)B (mSubAB), which was catalytically inactive, or as a control, PBS. Serine 272 and Histidine 89 are located in the amino acids of catalytic site, in a conserved sequence domain of subtilase family members [1]. The single amino acid mutant SubA(S272A)B has slight BiP-cleavage activity after prolonged incubation with Vero cells. Therefore, we used a double mutated toxin, SubA(S272A,H89A)B, which contains replacements of both catalytic serine and histidine residues. SubAB-treated mice showed decreased movements by 36 h after injection and died within 72 h. When a lower dose of SubAB (5 µg) was injected into mice, death was

observed between 5 to 7 days (data not shown). In contrast, as expected, mSubAB-treated mice were similar to controls. These data are consistent with a prior report, which showed that mice died at around 72 h after intraperitoneal injection of 10 µg (or 7.5 µg) of SubAB [13]. This lethal amount of SubAB is required for more than 100-fold that which would be required for Shiga-toxin 2 [14]. We investigated the quantify of SubAB produced by *E. coli* O29 in culture and associated with the organism. We found that 5×10^9 CFU bacteria produced about 2-3 µg of SubAB in culture supernatant and 2-3 µg of SubAB were associated with the bacteria (Fig. 1A). Both had BiP-cleavage activity in a HeLa cell assay (data not shown). Blood of SubAB-treated mice showed significant increases in BUN, AST, ALT, LDH, and WBC levels, and decreases in PLT and BS similar to a prior report [12]. In addition, we found a significant increase of AMY (Fig. 2, a), and significant decreases of TP and ALB (Fig.

2, b and c), compatible with pancreatic and hepatic abnormalities, respectively. T-Chol and TG at 48 h were significantly decreased (Fig. 2, d and e). Further, we observed that blood collected at 48 h from SubAB-treated mice did not coagulate, which was consistent with an increased PT and APTT (Fig. 2, e and f) and decreased Fib (Fig. 2, g).

2.2. ^{18}F -FDG PET imaging of SubAB-treated mice.

In SubAB-treated mice, WBC levels were significantly increased compared to mSubAB and control mice, consistent with the conclusion that SubAB induced an inflammatory response. To determine the sites of inflammation following injection of SubAB, we conducted ^{18}F -FDG PET imaging with SubAB-treated, mSubAB-treated, and control (PBS)-mice. Under inflammatory conditions, the affinity and expression of glucose transporters are increased by various cytokines and growth factors, and uptake of FDG is increased [15-18]. Usually, the physiological uptake of ^{18}F -FDG in the brain, muscle including myocardium, and genitourinary tract exceed those of other organs. Hepatic and splenic uptake is generally low grade and diffuse. Uptake in stomach and bowel is variable [17, 19, 20]. In small animals, warming,

fasting, and anesthesia influence uptake of ^{18}F -FDG [21]. Therefore, we performed ^{18}F -FDG PET in mice injected with SubAB, mSubAB, and PBS (control). At 24 h after injection, in SubAB-treated mice but not in mSubAB-treated or control mice, ^{18}F -FDG accumulated in the abdominal area corresponding to the small intestine (Fig. 3: circle). There were no obvious differences of ^{18}F -FDG uptake in kidney, liver, and spleen (Fig. 3). These data indicated that inflammation might be present in the small intestine of SubAB-treated mice.

2.3. SubAB induced severe hemorrhage in small intestine.

We did not recognize any macroscopic changes in brain, heart, lung, kidney, pancreas, and colon for up to 48 h in SubAB-treated, mSubAB-treated, and control mice. However, we observed small bowel hemorrhage at 48 h in wild-type SubAB-treated mice (Fig. 4A and B). In addition, liver of SubAB-treated mice was pale compared with mSubAB-treated or control mice (Fig. 4C), compatible with ischemic changes in the SubAB-treated mice. Spleen of SubAB-treated mice showed a dramatic reduction in the size as reported previously [12].

2.4. Histopathological analysis of SubAB-treated mice.

Organs (gut, kidney, brain, heart, lung, liver, spleen, and pancreas) were subjected to routine histological examination (HE staining) at 6 h, 24 h, and 48 h after injection of SubAB. We found significant bleeding into the small bowel of SubAB-treated mice. In the small intestine, inflammatory changes appeared as early as 6 h after wild-type SubAB injection and the damage was more severe with increasing time after injection (Fig. 5A). At 48 h after injection, neutrophil infiltration and associated hemorrhage were observed in the villus, muscularis mucosa, and muscle layer of the small intestine (Fig. 5A, d and i). These changes were observed in the upper part of the small intestine. No obvious differences between mSubAB-treated mice and control PBS-treated mice were seen at 48 h. In contrast to a prior report [12], we did not detect microvascular thrombosis and apoptosis in the kidney by TUNEL assay (data not shown). However, we found hyperplasia of the

juxtaglomerular apparatus and proliferation of glomerular mesangial cells in the kidney at 48 h similar to prior report [12].

There was no obvious histopathological damage in organs except small intestine in SubAB-treated mice (data not shown). Initial inflammation and degradation of the small intestine was observed at 6 h in the villus of SubAB-treated mice. Using cy3-labeled SubAB, we investigated which regions in the small intestine bound SubAB. In addition, cy3-labeled SubAB was able to bind to Vero cells [22] and heat-treated SubAB (100 °C, 10 min) lost binding activity [4]. Each section of the small intestine of control mice was incubated with cy3-SubAB or with heat-inactivated cy3-SubAB as described in Methods. As shown in Figure 5C, cy3-SubAB, but not heated cy3-SubAB, was bound strongly at the tip of the villus and at the serosa. We concluded that SubAB interacted with the villus region in the small intestine.

2.5. SubAB induced IL6 expression in small intestine of mice.

SubAB induced severe inflammatory changes in the small intestine (Figure 3, 4, 5A), consistent with the hypothesis that SubAB induced cytokine release. We investigated whether mRNA levels of cytokines were enhanced in the small intestine of

SubAB-treated mice. As IL1 α , IL1 β , IL4, IL6, IL10, IL12, IL17, IL22, IFN γ , TNF α and GM-CSF are known to major inflammatory cytokines [23], we investigated mRNA levels of these cytokines by RT-PCR analysis in SubAB-, mSubAB-, and control-treated mice at 6 h, 24 h, and 48 h after injection. There were no significant differences between SubAB-treated and control mice except for IL4, IL6 and IL12. IL6 mRNA was increased by SubAB, but not mSubAB in a time-dependent manner. In contrast, IL4 and IL12 mRNA was decreased by treatment with both SubAB and mSubAB (Fig. 6A). Next, we investigated the expression level of IL6 protein in the mice small intestine by ELISA. IL6 levels in SubAB-treated mice were significantly increased in a time-dependent manner (Fig. 6B).

3. Discussion

E. coli O29, isolated from a patient hospitalized with acute intestinal infection, produced SubAB and Stx2 but not Stx1[22]. Antibiotics frequently prescribed for the treatment of diarrheal diseases, often enhanced production of *E. coli* virulence factors [24]. We showed here that O29 (5×10^9 CFU) produced 2-3 μg of SubAB in culture supernatant and had similar amounts of toxin associated with bacteria. Antibiotic treatment after infection may release sufficient SubAB to induced severe tissue damage. We showed here that O29 (5×10^9 CFU) produced 2-3 μg of SubAB in culture supernatant and had similar amounts of toxin associated with bacteria. Antibiotic treatment after infection may release sufficient SubAB to induced severe tissue damage.

In this study, we showed that SubAB induced severe hemorrhage of the small intestine in mice. From macroscopic analysis, significant small bowel hemorrhage was observed

at 48 h, mainly in the upper region of the small intestine (Fig. 4); hemorrhage was not observed at 24 h. Previous reports did not observe intestinal damage and hemorrhage induced by SubAB [12]. They investigated that SubAB-caused extensive microvascular thrombosis and other histological changes in the brain, kidney and liver as well as splenic atrophy. We observed hyperplasia of the juxtaglomerular apparatus and proliferation of glomerular mesangial cells in the kidney, and splenic atrophy, in agreement with their results. However, we did not observe other histopathological change including apoptosis in these organs at 48 h; mice were dead within 72 h. Therefore, we consider intestinal hemorrhage to be the main cause of morbidity and mortality induced by administration of SubAB in mice. We wondered why no intestinal damage was observed by Wang et al [12]. One plausible explanation is differences in animal sensitivity to SubAB even with the same strain and similar age perhaps due to

genetic drift. RBC and Hb were not significantly decreased. Significant decrease of RBC and Hb may not necessarily be seen with rapid bleeding, since hemodilution does not occur immediately [25]. Ischemic change in the liver at 48 h in SubAB-treated mice was consistent with excessive bleeding and circulatory compromise, as were the elevations of AST and ALT. Elevated BUN levels were consistent with gastrointestinal bleeding due to ingested blood protein or kidney failure [26]. A coagulation disorder was also observed at 48 h in SubAB-treated mice. Although we did not observe small bowel hemorrhage at 6 h in SubAB-treated mice, thrombocytopenia was present and that may be an early feature of SubAB toxicity. Since $\alpha 2\beta 1$ integrin, a SubAB receptor, is present on the PLT surface [4, 27], thrombocytopenia may be a direct effect of toxin.

We observed that WBC was increased in SubAB-treated mice (data not shown), in agreement with previous studies [12]. These data suggest that SubAB induced an

inflammatory response in mice. To investigate the site of inflammation, ^{18}F -FDG PET images of SubAB-, mSubAB-, and PBS-treated mice were obtained at 24 h after injection. Significant uptake of ^{18}F -FDG was observed in the abdominal area corresponding to the gut, but not in the kidney, liver, and spleen (Fig. 3). By histopathology, inflammatory changes in the small intestine of SubAB-treated mice were evident as early as 6 h after injection. Neutrophil infiltration and hemorrhage in the small intestine progressed from villus to muscularis mucosa and muscle layer in a time-dependent manner (Fig. 5A). There was no obvious damage in organs except small intestine at 6 h, 24 h, and 48 h. Microvascular thrombosis in the brain, liver, and kidney were reported previously, [12], but were not observed in our studies (Fig. 5B). From PET imaging and histopathology, it is evident that SubAB was responsible for inflammation as well as hemorrhage in the small intestine.

Infection with STEC may result in HUS [28]. SubAB-induced neutrophil infiltration and apoptosis have been reported in the kidney [12]. However, in this study, severe kidney damage was not observed following injection of SubAB. By PAM staining, hyperplasia of the juxtaglomerular apparatus and proliferation of mesangial cells were seen in the kidney (Fig. 5B). CRE level in SubAB-treated mice, however, was not significantly increased (data not shown). Taken together, we conclude that SubAB induced renal damage, but not failure, and thus, differed from a prior study in the extent of renal involvement following SubAB treatment.

We confirmed that SubAB bound to small intestine using cy3-SubAB, which localized at the tip of villus and at the serosa (Fig. 5C). Thus, SubAB injected intraperitoneally may attach to the serosa directly and then be taken up by the systemic circulation. Based on biochemical and microscopic analysis, SubAB induced an inflammatory response in the

small intestine. We assessed changes of several cytokines by RT-PCR analysis, and found that IL6 mRNA and protein levels were significantly increased (Fig. 7A and 7B). IL4 and IL12 mRNA levels were decreased by both SubAB and mSubAB, suggesting that the effect was independent of the enzymatic activity of SubA and may have been induced by SubB. Various types of lymphoid and nonlymphoid cells (e.g., T cells, monocytes, fibroblasts) produce IL6 [29]. IL6 mediates inflammatory and stress-induced responses [30], leukocytosis and fever [31], and acts as a growth factor for renal mesangial cells [32]. Interestingly, the renal mesangial proliferation and hypoglycemia were seen in SubAB-treated mice at 48 h (Fig. 5B). IL6 overproduction has been shown to increase dramatically circulating insulin levels, resulting in hypoglycemia, as well as cause liver inflammation in mice [30]. In SubAB-treated mice, increased AST and ALT are consistent with liver damage. Hypoglycemia may have resulted from an increase in

insulin or hepatotoxicity. We propose that SubAB causes inflammatory injury, accompanied by IL6 production in the small intestine, and IL6 expression may have lead to mesangial proliferation and hypoglycemia. Thus, death in SubAB-treated mice may be associated with severe inflammation and hemorrhage of the small intestine, accompanied by coagulopathy and IL6 production.

4. Materials and Methods

4.1. Preparation of SubAB and catalytically inactive mutant, SubA(S272A,H89A)B.

Recombinant His-tagged SubAB was prepared as previously reported [22]. To replace active-site histidine (H89) with alanine in SubA, a QuikChange site-directed mutagenesis kit (Stratagene) was used. Plasmid encoding SubAB(S272A) [22] was used as a dsDNA template and primers were

5'-GTCTGAAGCTTTATATATTGCTGGTACTGCTATGGCTTCCC-3' and

5'-GGGAAGCCATAGCAGTACCAGCAATATATAAAGCTTCAGAC-3'

(mutated bases are underlined). PCR was performed following the manufacturer's instructions. Plasmids obtained from three colonies were sequenced and mutation was confirmed with an ABI 377 automatic sequencer. Mutant SubA (S272A, H89A)B was prepared using the same method as described for wild-type SubAB [22].

4.2. SubAB production in *E.coli* O29.

E. coli O29 was cultured in 5 ml of Brain Heart Infusion (BHI) medium for 14 h at 37 °C and then centrifuged at 17,400 x g for 1 min. The supernatant was saved for assay and the pellet was washed with ice-cold PBS and then treated with 5000 units of polymyxin B (Sigma) for 30 min at 37 °C. Bacterial extract was obtained by centrifugation at 5,000 x g for 10 min at 4 °C. The amount of SubAB was determined by Western blotting using anti-SubAB antibody. Standard samples of purified recombinant SubAB (0, 10, 25, 50 or 100 ng) were used in parallel to quantify the SubAB amount."

4.3. Animals.

Animal experiments were approved by Chiba University Institutional Animal Care and Use Committee, and the Committee of Care and Use of Laboratory Animals of the National Institute of Radiological Sciences, Chiba, Japan. Male BALB/c mice (Japan SLC, Shizuoka, Japan), 5-9 weeks old, were injected intraperitoneally (i.p) with 10 µg of purified SubAB or mutant SubAB (dissolved in 100 µl of PBS). As a control, mice received 100 µl of PBS.

4.4. Blood sampling and analysis.

At indicated times after SubAB injection, mice were anesthetized with pentobarbital (0.05 mg/g) by intraperitoneally injection. Blood samples were obtained via cardiac puncture and collected into lithium heparin or EDTA tubes. To determine prothrombin time (PT) and activated partial thromboplastin time (APTT), blood samples were

collected into 3.13% sodium citrate (dilution, 1:9) tubes. All blood tests (TP, total protein; ALB, albumin; T-Bil, total bilirubin; AST, aspartic aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; AMY, amylase; LIP, lipase; BUN, blood urea nitrogen; CRE, creatinine; T-Cho, total cholesterol; TG, triglyceride; Na, sodium; Cl, chlorine; K, potassium; Ca, calcium; BS, blood glucose; WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; PLT, platelet; Fib, fibrinogen; FDP, fibrin/fibrinogen degradation products; PT or APTT) were performed by Monolis, Inc (Tokyo, Japan).

4.5. Histopathology.

After blood sampling, organs were perfusion-fixed by injection with 20% formalin neutral buffer solution into heart (WAKO, Osaka, Japan), removed and fixed in 20 % formalin neutral buffer solution for 24 h at room temperature, embedded in paraffin,

sectioned, and stained with hematoxylin-eosin (HE) and, for renal histopathology, with periodic acid-methenamine-silver (PAM) stain, and examined by light microscopy.

4.6. Fluorescence immunostaining.

SubAB was labeled with cy3 using a Fluorolink-Ab Cy3 labeling kit (Amersham Pharmacia Biotech, Ltd.) as described previously [22]. Small intestines of control mice were fixed in 20 % formalin neutral buffer solution for 24 hours at room temperature, embedded in paraffin, and sectioned. Sections (4 μm) were placed on salinized slides. Paraffin sections were deparaffinized and rehydrated, and antigens were retrieved by incubation of slides in 0.4 $\mu\text{g}/\mu\text{l}$ proteinase K (Dako) for 10 min at room temperature. After washing with PBS, slides were subsequently blocked in 10 % normal goat serum (Invitrogen, CA, USA) for 30 min at room temperature. After slides were washed twice with PBS for 5 min, each slide was incubated with heated cy3-SubAB (100 $^{\circ}\text{C}$, 10 min)

as a negative control or with cy3-SubAB for 30 min at room temperature. Washed slides were mounted in ProLong Gold Antifade Reagent with DAPI (Invitrogen) and examined by confocal microscopy (FLUOVIEW FV10i, OLYMPUS).

4.7. RT-PCR.

Total RNA was extracted from small intestine at 6 h, 24 h and 48 h after SubAB, mSubAB, or control PBS injection using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 5 µg of total RNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, UK). cDNA was amplified in a 50 µl PCR mixture according to the manufacturer's protocol (TaKaRa Bio, Shiga, Japan). The PCR conditions were as follows: 30 cycles of 98 °C for 10s, 55 °C for 30s, and 72 °C for 60s. Primers for PCR are given in Table 1. PCR products were subjected to electrophoresis on 2 % agarose

gels containing ethidium bromide, and the bands were visualized under ultraviolet (UV) light.

4.8. IL6 levels.

To determine IL6 levels, after intraperitoneal injection of SubAB, mSubAB, or PBS as a control, small intestine was removed at the indicated times and homogenized in ice-cold PBS buffer containing protease inhibitor cocktail (Roche Diagnostics). The homogenates were centrifuged at 15,000×g for 2 min at 4 °C. Supernatants (10 µg in 100 µl) were analyzed for IL6 with Mouse IL-6 ELISA MAX (BioLegend, San Diego, USA) according to the manufacturer's protocol. Optical densities were measured at 450 nm on an iMark microplate reader and the amount of IL6 was quantified with standard curves using mice IL6 standard by Microplate Manager 6 Software (BIO-RAD).

4.9. ^{18}F -fluoro-2-deoxy-D-glucose (FDG) positron emission tomography (PET) imaging.

At 24 h after i.p injection of wild-type SubAB (10 μg in PBS), mutant SubAB (10 μg in PBS) or PBS, and following an overnight fast, mice were injected by tail vein with ~ 4 MBq of ^{18}F -FDG (Nihon Medi-Physics, Tokyo, Japan). At 50 min after ^{18}F -FDG injection, a 10-min emission scan was performed using a dedicated small animal PET system (Inveon, Siemens Medical Solutions, Malvern, PA) under 1.5–2% isoflurane anesthesia during the entire scanning period. Mice were heated by a lamp during the PET scan to maintain body temperature. Images were reconstructed using a 3D maximum *a posteriori* (18 iterations with 16 subsets, $\beta = 0.2$ resolution) without attenuation correction using Inveon Acquisition Workplace software (Siemens Medical Solutions). PET images were quantified by standardized uptake value (SUV), which was calculated using the following

formula: $SUV = \text{regional radioactivity concentration (Bq/mL)} / \text{injected dose (Bq)} \times \text{body weight (g)}$, where radioactivity was decay-corrected from the delay between injection and image acquisition.

4.10. Statistical analysis.

Statistical analysis was performed using Prism software (version 5.0; GraphPad).

Data are presented as mean $\pm$ SEM. The differences were analyzed using Student's *t* test.

Results were considered significant when $P < 0.05$ was obtained.

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Figure legends

Figure 1. SubAB levels in *E. coli* O29 culture supernatant or associated with bacteria.

(A) The indicated amounts of O29 culture supernatant (Sup), polymyxin B bacterial extract (bac) or purified recombinant SubAB as a standard were analyzed by SDS-PAGE in 15 % gel, and transferred to PVDF membranes, which were incubated with anti-SubAB antibody followed by ECL detection. Lane 1 and 5, 1 μ g; lane 2 and 6, 0.5 μ g; lane 3 and 7, 0.25 μ g; lane 4 and 8, 0.1 μ g; lane 9, 100 ng; lane 10, 50 ng; lane 11, 25 ng; lane 12, 10 ng; lane 13, PBS as a control. Data are representative of two separate experiments.

Figure 2. Effect of SubAB on blood laboratory values.

Blood was collected from control or treated mice at the indicated times after the intraperitoneal injection of SubAB (10 μ g), mSubAB (10 μ g) or PBS (n=3 for each

group). Differences between control and treated mice were analyzed using Student's *t*

test: **P*<0.05; ***P*<0.01; *** *P*<0.001.

a) AMY, amylase; b) TP, total protein; c) ALB, albumin; d) T-Cho, total cholesterol; e)

TG, triglyceride; f) PT, prothrombin time; g) APTT, activated partial thromboplastin

time.

Figure 3. Coronal images of ¹⁸F-FDG-PET in SubAB-treated mice.

At 24 h after administration of SubAB (n=2), mSubAB (n=2) or PBS (n=1), mice were

injected via the tail vein with ~4 MBq of ¹⁸F-FDG and then ¹⁸F-FDG-PET imaging

studies were obtained. SubAB-treated (a and b), mSubAB-treated (c and d), and control

(PBS) (e) mice. The abdominal area is shown by a circle. Uptake of ¹⁸F-FDG (arrowhead)

was observed in the abdominal area corresponding to the gut. Physiological uptake of

^{18}F -FDG in the brain, muscle including myocardium, and genitourinary tract exceeded those of other organs. SUV: standardized uptake value.

Figure 4. Anatomy of SubAB-treated mice.

Mice were sacrificed at 48 h after injection of SubAB, mSubAB, or PBS. (A)

Abdominal exposure of control, SubAB- or mSubAB-treated at 48 h. Right panels show the gastrointestinal tract (B) or liver (C) from mice in panel A. Small bowel hemorrhage and liver ischemia were seen at 48 h after SubAB injection. Data are representative of separate experiments (n=7 in each group).

Figure 5. Microscopic analysis in SubAB-treated mice.

(A) Hematoxylin-eosin (HE) staining of small intestine in SubAB, mSubAB, and

control mice. Control (a), SubAB-treated at 6 h (b), SubAB-treated at 24 h (c),

SubAB-treated at 48 h (d), and mSubAB-treated at 48 h (e). (f-j: enlarged image of a-e)

n=3 for each group. (B) Periodic acid-methenamine-silver (PAM) stain of kidneys in SubAB and control mice. PAM staining of control mice (a) and SubAB-treated at 48 h (b). (c) and (d): enlarged image of square of (a) and (b). Hyperplasia of the juxtaglomerular apparatus and proliferation of glomerular mesangial cells were observed in SubAB-treated mice (b and d: arrow), but not control (a and c: arrow). n=3 for each group. (C) Detection of cy3-SubAB in small intestine by confocal microscopy. Sections of small intestine from control mice were incubated with cy3-labeled SubAB or heated cy3-SubAB as a negative control for 30 min at room temperature. In small intestine (a-h), DIC (a and e), DAPI (b and f), heated cy3-SubAB (c), native cy3-SubAB (g), and merged image (d: b and c, h: f and g) show SubAB attached to the tip of villus and serosa (arrow).

Figure 6. Effect of SubAB on various cytokines in small intestine.

(A) Cytokine mRNA levels in the small intestine of SubAB-treated mice.

Mice were treated with PBS, SubAB, or mSubAB for the indicated times. Total RNA was extracted from the intestine, and mRNA levels of indicated genes were analyzed by semiquantitative RT-PCR, as described in Materials and Methods. Primers are shown in Table 1[33,34]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. n= 3 for each group.

(B) Protein expression level of IL6 in the small intestine of SubAB-treated mice. After administration of SubAB, mSubAB, or PBS to mice, small intestine was removed at the indicated time points and homogenized. After centrifugation, the supernatants were collected as described in Materials and Methods. 10 µg of each sample were analyzed for IL6 with mouse IL6 ELISA Data presented are from separate ELISAs on duplicate samples from one set of groups at indicated times (n=3 for each group). Differences

between control and treated mice were analyzed using Student's *t* test: * $P < 0.05$;

** $P < 0.01$; *** $P < 0.001$.

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Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5A

Fig. 5B

Fig. 5C

Fig. 6A

Fig. 6B

Table 1. Primers for RT-PCR

primer		sequence(5'-3')	Position	Size of Product (bp)
IL1 α	forward	CGTCAGGCAGAAAGTTTGTCA	393-412	376
	reverse	GTGCACCCGACTTTGTTCTT	768-749	
IL1 β	forward	CAGGCAGGCAGTATCACTCA	180-199	350
	reverse	AGGCCACAGGTATTTTGTCTG	529-510	
IL4	forward	CATCCTGCTCTTCTTTCTCG	114-133	377
	reverse	TGATGCTCTTTAGGCTTTCC	490-471	
IL6	forward	GCTATGAAGTTCCTCTCTGC	29-48	536
	reverse	AGCTTATCTGTTAGGAGAGC	564-545	
IL10	forward	AGCCGGGAAGACAATAACTG	145-164	407
	reverse	TTCATGGCCTTGTAGACACC	551-532	
IL12	forward	TCCAGCATGTGTCAATCACG	153-172	466
	reverse	CCTTGTCTAGAATGATCTGC	618-599	
IL17	forward	CTGGAGGATAAACTGTGAGAGT	142-164	226
	reverse	TGCTGAATGGCGACGGAGTTC	368-348	
IL22	forward	CATGCAGGAGGTGGTACCTT	342-361	250
	reverse	CAGACGCAAGCATTTCTCAG	538-519	
IFN γ	forward	TCTGAGACAATGAACGCTAC	101-120	457
	reverse	GCTTCCTGAGGCTGGATTCC	557-538	
TNF α	forward	ACGGCATGGATCTCAAAGAC	353-372	324
	reverse	CGGACTCCGCAAAGTCTAAG	676-657	
GM-CSF	forward	CCGTAGACCCTGCTCGAATA	83-102	161
	reverse	CCGTAGACCCTGCTCGAATA	243-224	
GAPDH	forward	TGAGGCCAGTGCTGAGTATG	258-277	255
	reverse	CCTTCCACAATGCCAAAGTT	512-493	