Microbiol. Immunol., **46**(4), 249−255, 2002

Inhibition by Apple Polyphenols of ADP-Ribosyltransferase Activity of Cholera Toxin and Toxin-Induced Fluid Accumulation in Mice

Takao Saito[1, 2], Masami Miyake[1], Masamichi Toba[2], Hiroshi Okamatsu[2], Seiichi Shimizu[3], and Masatoshi Noda*[, 1]

[1]*Department of Molecular Infectiology, Graduate School of Medicine, Chiba University, Chiba, Chiba 260−8670, Japan,* [2]*Otsu Nutraceuticals Research Institute, Otsuka Pharmaceuticals Co., Ltd., Otsu, Shiga 520−0002, Japan, and* [3]*P-14 Project, Otsuka Pharmaceuticals Co., Ltd., Osaka, Osaka 540−0021, Japan*

Received October 12, 2001. Accepted January 5, 2002

***Abstract*: The effects of crude polyphenol extracted from immature apples on the enzymatic and biological activities of a cholera toxin (CT) were investigated. When the apple polyphenol extract (APE) was examined for properties to inhibit CT-catalyzed ADP-ribosylation of agmatine, it was found that APE inhibited it in a dose-dependent manner. The concentration of APE to inhibit 50% of the enzymatic activity of CT (15 μg/ml) was approximately 8.7 μg/ml. The APE also diminished CT-induced fluid accumulation in two diarrhea models for *in vivo* mice. In the ligated ileum loops, 25 μg of APE significantly inhibited fluid accumulation induced by 500 ng of CT. In a sealed mouse model, even when APE was administered orally 10 min after a toxin injection, fluid accumulation was significantly inhibited at a comparable dosage. Lineweaver-Burk analysis demonstrated that APE had negative allosteric effects on CT-catalyzed NAD: agmatine ADP-ribosyltransferase. We fractionated the APE into four fractions using LH-20 Sephadex resin. One of the fractions, FAP (fraction from apple polyphenol) 1, which contains non-catechin polyphenols, did not significantly inhibit the CT-catalyzed ADP-ribosylation of agmatine. FAP2, which contains compounds with monomeric, dimeric, and trimeric catechins, inhibited the ADP-ribosylation only partially, but significantly. FAP3 and FAP4, which consist of highly polymerized catechin compounds, strongly inhibited the ADP-ribosylation, indicating that the polymerized structure of catechin is responsible for the inhibitory effect that resides in APE. The results suggest that polymerized catechin compounds in APE inhibit the biological and enzymatic activities of CT and can be used in a precautionary and therapeutic manner in the treatment of cholera patients.**

***Key words*: Apple polyphenols, Cholera toxin, ADP-ribosyltransferase, Fluid accumulation**

Cholera is a disease caused by the colonization of *Vibrio cholerae* in the upper intestinal tract, which causes massive secretory diarrhea, severe dehydration, and circulatory collapse (16). Severe dehydration is responsible for 20−45% of the deaths of patients who do not receive rehydration therapy (2). According to a report of the World Health Organization (21), 254,310 cases and 9,175 deaths due to cholera were reported worldwide in 1999. Cholera continues to be a major public health problem in developing countries. In Japan, cases of imported infection have been reported by the Ministry of Health and Welfare every year from 1990 through 1999 (5).

A major virulence factor of *V. cholerae* is the cholera toxin (CT), a proteinaceous toxin produced by the bacterium (1). The toxin consists of one enzymatic A subunit and five B subunits. The A subunit has ADP-ribosyltransferase (ART) activity, which mono-ADP-ribosylates a mammalian GTP-binding regulatory protein, Gsα, of the adenylate cyclase system. The A subunit associates with the homo-pentameric B-oligomer, which

**Address correspondence to* Dr. Masatoshi Noda, Department of Molecular Infectiology, Graduate School of Medicine, Chiba University, 1−8−1 Inohana, Chuo-ku, Chiba, Chiba 260−8670, Japan. Fax: 81−43−226−2049. E-mail: noda@med.m.chiba-u.ac.jp

Abbreviations: ADP, adenosine 5′-diphosphate; APE, apple polyphenol extract; ART, ADP-ribosyltransferase; CT, cholera toxin; FA, fluid accumulation; FAP, fraction from apple polyphenol; GTP, guanosine 5′-triphosphate; K_m, Michaelis constant; NAD, nicotinamide adenine dinucleotide; SD, standard deviation; TLC, thin-layer chromatography.

mediates the binding of the toxin to a specific receptor, G_{M1} monosialoganglioside, on the surface of target eukaryotic cells (3). The bound toxin is then endocytosed in endosomal vesicles and transported intracellularly through the Golgi apparatus to the endoplasmic reticulum, where the A subunit is translocated out of the vesicle into cytosol (11). It is believed that the translocated A subunit moves to the inner surface of the plasma membrane and ADP-ribosylates Gsα, resulting in constitutive activation of the adenylate cyclase. The intoxication of intestinal epithelial cells by the toxin causes the accumulation of intracellular cAMP, which stimulates chloride secretion from and inhibits neutral sodium chloride absorption into intestinal mucosa, consequently leading to the severe secretory diarrhea observed in cholera patients (1, 3).

Since CT is a major factor responsible for the induction of diarrhea in cholera, it is expected that inactivation of CT-derived ADP-ribosyltransferase activity in the intestinal tract would block the onset of the disease, leading to a decrease in the mortality rate of cholera patients (17). Toda et al. (19) have reported that tea polyphenol inhibited the fluid accumulation induced by CT in sealed adult mice. Effects of other polyphenols, however, were not elucidated until recently. In the present study, we describe how a crude polyphenol fraction extracted from immature apples inhibits both the enzymatic and biological activities of CT. This suggests the possibility that oral administration of an apple polyphenol can be used in cholera patients as a precautional and therapeutic procedure.

Materials and Methods

Chemicals. Crude apple polyphenol extract (Applephenon SH) was purchased from Nikka Whisky Distilling Co., Ltd. (+)-Catechin, phloretin, phloridzin, *p*-coumaric acid, and chlorogenic acid were from Sigma-Aldrich Japan K.K. Procyanidin B1, procyanidin B2, procyanidin C1, and (−)-epicatechin were from the Funakoshi Co., Ltd.

CT was purchased from the List Biological Laboratories; [adenine-U-^{14}C]NAD (250–300 mCi/mmol), from Amersham Pharmacia Biotech K.K.; AG1-X2 resin (200–400 mesh), from Nippon Bio-Rad Laboratories; and pentobarbital, from the Dainippon Pharmaceutical Co., Ltd. All other reagents were either from Wako Pure Chemical Industries, Ltd. or from Sigma-Aldrich Japan K.K.

Preparation of FAPs. Fractionation of APE into four FAPs (fractions from apple polyphenol) was kindly carried out by Dr. Junei Kinjo (Fukuoka University) by the established procedure (18). Briefly, APE was dissolved in distilled water and applied to a Sephadex LH-20 (Amersham Pharmacia Biotech K.K.) column. By elution with methanol, three distinct fractions, FAP1, FAP2, and FAP3, were recovered from the column in this order. An additional elution with acetone-water (1:1) resulted in the recovery of FAP4. The polyphenol contents of these fractions were identified by thin-layer chromatography (TLC) using Kieselgel 60 as a TLC plate and Benzene-HCOOEt-HCOOH (1:7:1) as a developing system (4). The indications were that FAP1 mainly contained non-catechin polyphenols and that FAP2, FAP3, and FAP4 contained monomeric to trimeric catechins, oligomeric catechins, and polymeric catechins, respectively. From 300 mg of apple polyphenol, 105 mg, 77 mg, 37 mg, and 57 mg of evaporated FAP1, FAP2, FAP3, and FAP4 were obtained, respectively.

ADP-ribosyltransferase activity. To quantify the ADP-ribosyltransferase activity of CT, an agmatine assay was performed as described (13). The reaction was started by the addition of a reaction buffer containing [^{14}C]NAD to 3 μg of CT and several amounts of APE, FAPs, or other polyphenols, in a total volume of 200 μl. After incubation for 3 hr at 30 C, a 100 μl portion of the reaction mixture was applied to an AG1-X2 column (5×45 mm). The unbound materials were eluted with distilled water and collected in scintillation vials. The radioactivity in the eluate was measured with a liquid scintillation counter.

Fluid accumulation in the ileal loop assay. A mouse ileal loop assay was performed as described previously (6). Four-week-old male ICR mice (JAPAN SLC., Inc.) weighing 20 to 25 g were used. The mice were starved overnight with free access to water and anesthetized with sodium pentobarbital, and their intestines were exteriorized through a midline incision. One ligated intestinal segment (loop) about 2 cm long was created per intestine. Five hundred ng of CT and/or APE (total volume 100 μl) was simultaneously injected into the loop. In 6 hr, the mice were killed, and the loop was removed from the carcass. The degree of fluid accumulation was expressed as the ratio of loop content (weight) to loop length.

Fluid accumulation in the sealed mice model. The mice were starved for 20 hr with free access to water before the experiments. Each mouse was intragastrically given 0.2 ml of a 3% sodium-hydrogen-carbonate solution containing 20 μg of CT. In 10 min, 0.1 ml of test agents was intragastrically administered to the mice. After a 6-hr incubation, the mice were sacrificed. The whole intestine (from the pyloric valve to the rectum) was resected and weighed. The fluid accumulation (FA) value was calculated by the following formula:

FA value $= A \div (B - A) \times 1{,}000$, where A represents

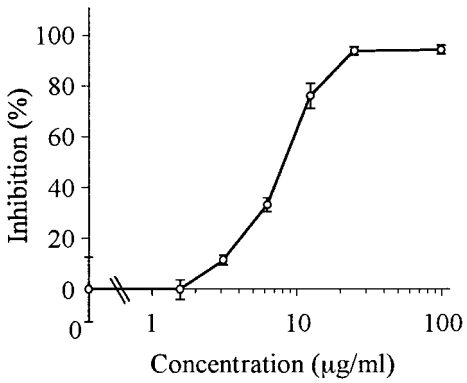

Fig. 1. Inhibitory effects of APE on CT-catalyzed ADP-ribosylation. Three μg of CT (15 μg/ml) was incubated for 3 hr at 30 C with agmatine in the presence of the indicated concentrations of APE. Values represented are the mean and standard deviations from 3 determinations.

the weight of the excised gut and *B* represents the body weight of the mouse.

Statistical analysis. Data were expressed as means± SD, and the differences in the groups were analyzed using one-way ANOVA with Dunnett's multiple compared test. A *P* value of <0.05 was considered statistically significant. All statistical analyses were performed using SAS ver.6.12 (SAS Institute).

Results

Inhibitory Effect of APE on CT-Catalyzed ADP-Ribosylation of Agmatine

The enzymatic A subunit of CT catalyzes the transfer of ADP-ribose from NAD to agmatine and other simple guanidino compounds (13). We examined the effect of APE on the CT-catalyzed ADP-ribosylation of agmatine. When APE was added in the assay mixture, it inhibited the ADP-ribosylation in a dose-dependent manner (Fig. 1). This result indicates that APE inhibits the enzymatic activity of CT. Under this condition, the concentration of APE to inhibit 50% of CT-catalyzed ADP-ribosylation was estimated to be 8.7 μg/ml.

Effect of APE on CT-Induced Fluid Accumulation in Mice

Since one of the biological activities of CT is the induction of fluid accumulation in the ileal tract, the inhibitory effects of APE on the CT-induced fluid accumulation in a mouse intestinal-loop assay were investigated. When a 500 ng/loop of CT was inoculated, approximately 17 mg of fluid accumulation/cm in the loop was observed, indicating the diarrhegenic activity of CT in this animal model. When APE at concentrations higher than 25 μg/ml was simultaneously injected into the loop, the fluid accumulation was significantly reduced

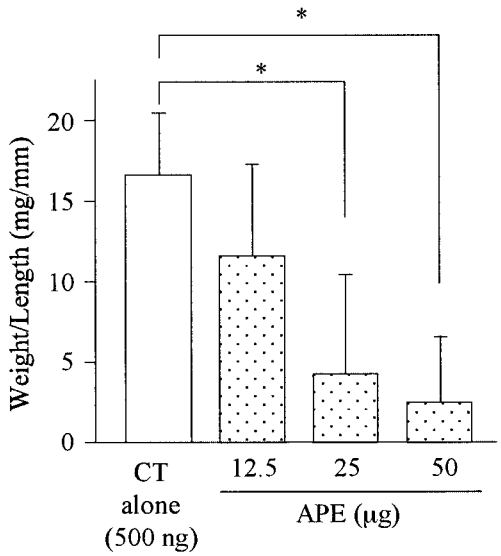

Fig. 2. Inhibitory effects of APE on the CT-induced fluid accumulation in the mouse ileal loop assay. Each loop was injected with 0.1 ml of a solution containing 500 ng of CT and/or 12.5 μg, 25 μg, or 50 μg of APE. In 6 hr, mice were sacrificed, and the loop was removed from the carcass. The degree of fluid accumulation was expressed as the ratio of loop content (weight) to loop length. Values represented are the mean and standard deviations from five animals. These values were compared with CT alone by the Dunnett's two-tailed test (*: $P<0.01$).

(Fig. 2). The result indicates that APE not only inhibits the enzymatic activity of the CT but also disturbs its biological activity *in vivo*.

The inhibitory effect of APE on CT-induced fluid accumulation was also confirmed in the sealed mouse model, in which toxin and APE were administered *per os*. In this model, CT was intragastrically delivered through the mouth and esophagus using thin tubing. At 10 min after toxin administration, APE was delivered by the same route. When 10 mg or 5 mg of APE was given to the mice, the FA values decreased by 60% of the value from the positive control (CT alone, Fig. 3). The degree of the decrease was statistically significant. The results indicate that, even when APE ingestion was made 10 min after CT administration, it still inhibited CT-induced fluid accumulation.

Qualification of Inhibitory Components in APE

Kanda and his collaborators reported the polyphenol composition in APE, which they had analyzed by high performance liquid chromatography (9). According to their report, APE consists of chlorogenic acid (20%, dry weight/dry weight), phloridzin (4%), phloretin (1%), *p*-coumaric acid (1%), caffeic acid (3%), (−)-epicatechin (6%), (+)-catechin (0.5%), procyanidin B1 (3%), procyanidin B2 (7%), oligomeric and polymerized catechins (40%), and unknown residual compounds (15%).

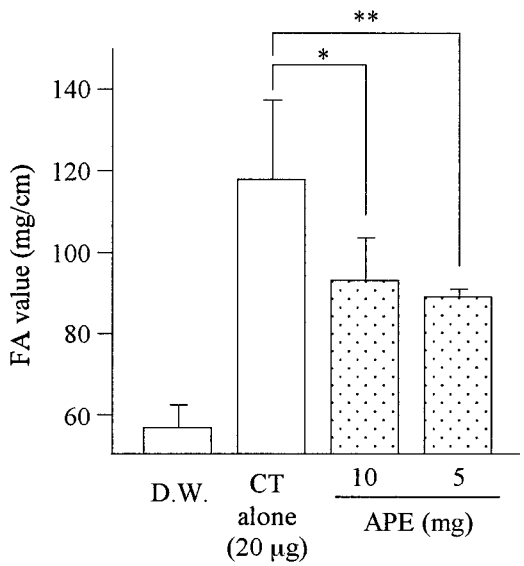

Fig. 3. Inhibitory effects of APE on CT-induced fluid accumulation (FA) in sealed mice model. After administration of CT for 10 min, the mice were given 5 mg or 10 mg of APE. After a 6-hr incubation, the mice were sacrificed. The whole intestine was resected and weighed. The FA value was determined as a ratio of the body weight minus the intestine weight to the intestine weight. Values represented are the mean and standard deviations, *: $P<0.05$).

In order to identify which polyphenols in APE are mainly responsible for the inhibitory effect on CT-catalyzed ADP-ribosylation, we fractionated the APE-derived polyphenols into four different fractions and compared their ability to inhibit the ADP-ribosyltransferase activity of CT. One of the fractions, FAP1, which contains non-catechin-type polyphenols such as chlorogenic acid, phloridzin, phloretin, *p*-coumaric acid, and caffeic acid, did not inhibit the ADP-ribosylation of agmatine at 25 μg/ml (Table 1). The inhibitory activity of FAP2, which contains mono- to trimeric catechins [(−)-epicatechin, (+)-catechin, procyanidin B1, procyanidin B2, and procyanidin C1], was significantly higher than that of FAP1, but only 40% of the transferase activity was inhibited by the fraction. FAP3 and FAP4, which contain highly polymerized (tetrameric or higher) catechin compounds, strongly inhibited the enzymatic activity of CT, suggesting that highly polymerized catechins are the major inhibitory components in APE (Table 1). In the next step, we obtained the isolated polyphenols from commercial sources and examined whether they inhibited CT-catalyzed ADP-ribosylation. The inhibitory activity of non-catechin polyphenols such as chlorogenic acid, phloridzin, phloretin, and *p*-coumaric acid at 25 μg/ml was only weak: 12.7±5.5%, 4.6±3.8%, 8.4±6.6%, 2.8±5.0%, and 5.3±10.1% of the ADP-ribosylation was inhibited, respectively. Monomeric catechins, such as (−)-epicatechin and (+)-catechin, did not inhibit it significantly, but procyanidin B1, procyanidin B2 (dimeric catechins), and procyanidin C1 (trimeric catechin) showed relatively higher inhibitory activity at the same concentration (2.4±3.3%, 0.1±5.3%, 17.4±12.7%, 9.8±10.5%, and 25.4±5.5%, respectively). Based on these results, together with the polyphenol composition in APE, it is concluded that highly polymerized catechins in FAP3 and FAP4 are the major inhibitory polyphenols in APE and that the non-catechin type and monomeric catechins exhibit a very slight effect, if any, on CT-catalyzed ADP-ribosylation.

Table 1. Qualification of inhibitory fractions in APE

Sample	Inhibition (%)	Components
Control	0.0±4.4	—
APE	95.4±0.7	—
FAP1	3.5±5.2	Non-catechin polyphenols
FAP2	39.4±5.8	Mono- to trimeric catechins
FAP3	98.3±1.5	Oligomeric catechins
FAP4	95.5±0.3	Polymeric catechins

CT at 15 μg/ml was incubated for 3 hr at 30 C with agmatine and [^{14}C]NAD in the presence or absence of a concentration (25 μg/ml) of APE and FAPs. The values represented are the mean and standard deviations from three determinations.

Effect of APE on the Kinetics of ART Activity

To determine how APE inhibits the enzymatic activity of CT, a Lineweaver-Burk analysis was performed (14). APE had no effect on the K_m for NAD but significantly reduced the maximal velocity (Fig. 4A). Unlike the case with NAD, APE significantly increased the K_m for agmatine without altering the maximal velocity (Fig. 4B). These data suggest that APE had a different effect on the ART activity of CT when agmatine or NAD was examined. We concluded from these data that APE has a negative allosteric effect on CT.

Discussion

In the present study, we demonstrated that APE, the polyphenol extract from immature apples, inhibited CT-catalyzed ADP-ribosylation and CT-induced fluid accumulation in mice. It also turned out that the inhibitory substances in the extract were highly polymerized catechin compounds, although dimeric and trimeric catechins also showed lower inhibitory activity. Although these observations may simply suggest that APE inhibits CT-induced fluid accumulation by disturbing the A subunit-residing ADP-ribosyltransferase activity, the precise mechanism for how polymerized catechins inactivate the enzymatic activity of CT is not fully known at this moment. Our preliminary observation, however, suggests that the catechins tightly bind to CT, resulting in irre-

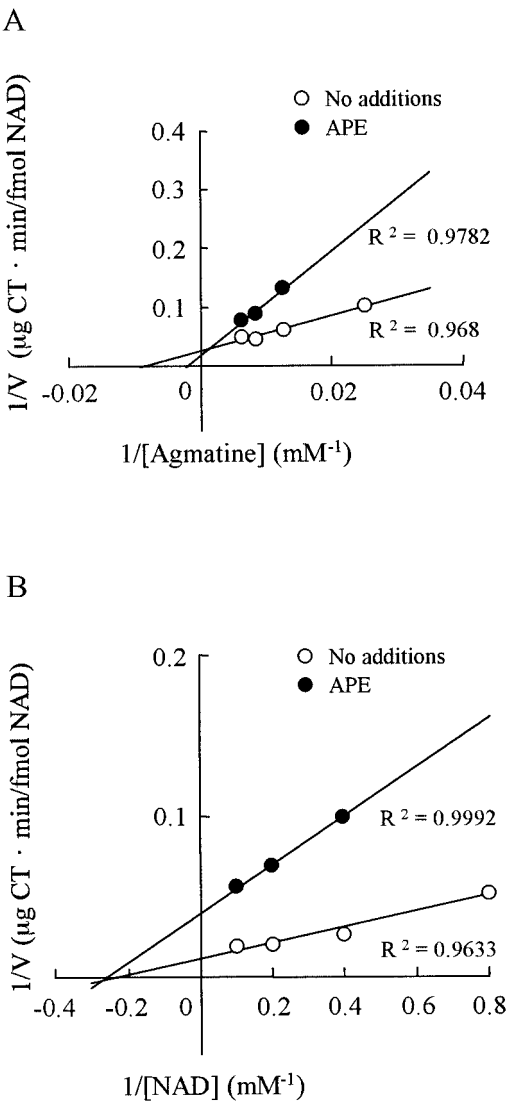

Fig. 4. Effect of APE on the kinetics of ART activity. Assay contained A: 15 μg/ml CT, 100 μM NAD, and the indicated concentrations of agmatine, B: 15 μg/ml CT, 20 mM agmatine, and the indicated concentrations of NAD. (○) no additions; (●) 8.7 μg/ml APE.

versible inactivation of the toxin (our unpublished observation). An additional explanation for the *in vivo* effect of APE is that APE not only inhibits the transferase activity of CT but also modifies a function of the intestinal mucosa, protecting it from a secretory response against the diarrhegenic bacterial toxin. It has been reported that proanthocyanidins affect the intestinal mucosal function, resulting in a reduction of CT-induced fluid accumulation (7). Since proanthocyanidins are categorized from their structure into the same group as the catechin polymer in APE (22), it is probable that the polymerized catechins in APE also show similar protective effects on the host intestine. Our hypothesis that APE shows a protective effect on intestinal mucosa is supported by the observation that catechin compounds from another plant source, which showed potent inhibition of the transferase activity, disturbed CT-induced fluid accumulation to a much smaller degree than APE (our unpublished observation). Future studies should examine whether APE has the potential to act on intestinal mucosal function.

The effective doses of APE to inhibit CT-induced fluid accumulation in two bioassays differed considerably (Figs. 2 and 3). The different effectiveness can be partly explained by the difference in CT amounts used in the assays; 40 times more CT was administered per mouse in sealed mice model. It is considered that additional factors that caused the difference are route and timing of administrations. In ileal loop assay, CT and APE were injected simultaneously into the intestinal lumen where the toxin acts. On the contrary, in sealed mice model the toxin and APE were injected in the gastric tract, which is generally filled with ingested food even after 48-hr starvation (our observation). The toxin and polyphenols in gastric lumen should be then delivered to intestinal tract, together with gastric and intestinal contents mixed with digestive juice. Thus, the degradation and/or modification of APE might have occurred under the condition, resulting in the lower efficiency of APE to inactivate CT in the sealed mice model.

Cholera patients are treated with an oral rehydration solution, which relieves life-threatening dehydration and reduces the mortality rate of the disease (16). Polyphenols that inhibit CT-induced fluid accumulation may exert an additional anti-diarrheal effect. For this purpose, other investigators have reported that tea polyphenol has an inhibitory effect on CT-induced fluid accumulation in sealed adult mice and the ligated intestinal loops of rabbits (19) and that it has a protective effect on gnotobiotic mice infected with the *Escherichia coli* O157:H7 strain (8). Similar to tea polyphenols, APE is frequently used as a food additive. Therefore, simultaneous ingestion of APE with the oral rehydration solution would be expected to reduce the CT-induced fluid accumulation and protect intestinal mucosa from a secretory response. The minimal APE concentration to inhibit more than 90% of CT-catalyzed ADP-ribosylation was estimated to be 25 μg/ml (Fig. 1). From a previous report that describes the detection of CT in stools of cholera patients (15), we conjectured that the concentration of CT in the intestine is between 26 pg/ml and 100 ng/ml. Considering these data, more than 200 ng/ml of APE would inactivate CT in the stools of the cholera patients. It has been reported that stool excretion in cholera patients is generally less than 10 liters/day but reaches 30 liters/day in severe cases (12, 20). Thus, if a cholera patient could take more than 30 mg of APE in a

day, and if the ingested APE could be kept in the patient's gut lumen at a concentration of more than 1 μg/ml, the APE might neutralize CT, resulting in the reduction of water excretion from the patient's bowel. APE itself is not toxic to humans and can be administered without apparent side effects at a dose of 10 mg/kg/day (10). Considering all the above-mentioned results, we think that APE could be used on cholera patients as a precautionary or therapeutic agent. Further studies with cholera patients are necessary in order to confirm the efficacy of APE for these purposes.

Although APE was similar to the tea polyphenols in that both polyphenol preparations exhibited a protective effect against the diarrhegenic activity of CT, the difference in their polyphenol compositions should be noted. Toda and her collaborators, who have reported the inhibitory activity of tea polyphenol on CT-induced fluid accumulation in mice and rabbits, described the polyphenol composition of their tea catechin preparation as follows (19): (−)-epigallocatechin gallate (59%, dry weight/dry weight), (−)-epigallocatechin (19%), (−)-epicatechin gallate (14%), (−)-epicatechin (6%), and (+)-gallocatechin (2%). This suggests that monomeric catechins and their galloyl derivatives would be the major inhibitory substances in tea polyphenols. Unlike the tea polyphenols, the major inhibitory polyphenols in APE were considered to be polymerized catechins in this study. Therefore, it is of interest how these structurally unrelated polyphenols show inhibitory activity on the same bacterial toxin. Future studies may shed light on the structure-function relationship of the inhibitors for bacterial ADP-ribosyltransferase.

We thank Dr. Iwao Kato and Dr. Yoshifumi Takeda for their participation in helpful discussions. We are grateful to Dr. Jyunei Kinjo for the fraction of APE into four FAPs. In addition, we thank Ms. Emi Utsuno and Ms. Michiko Hatano for their technical assistance.

This work was supported by Grants-in-Aid of Scientific Research (03454180, 04304032, 05271205, 06264204, and 08770184) from the Ministry of Education of Japan and Grants for International Health Cooperation Research (11A-1-1999, 11A-1-2000) from the Ministry of Health and Welfare, Japan.

References

1) Asakura, H., and Yoshioka, M. 1994. Cholera toxin and diarrhoea. J. Gastroenterol. Hepatol. **9**: 186−193.
2) Chen, L., Rahman, M., and Sarder, A.M. 1980. Epidemiology and causes of death among children in a rural area of Bangladesh. Int. J. Epidemiol. **9**: 25−33.
3) Fishman, P.H. 1990. Mechanism of action of cholera toxin, p. 127−140. *In* Moss, J., and Vaughan, M. (eds), ADP-ribosylating toxins and G proteins, American Society for Microbiology, Washington, D.C.
4) Furuichi, E., Nonaka, G., Nishioka, I., and Hayashi, K. 1986. Isolation and structures of procyanidins (condensed tannins) from *Rhaphiolepis umbellata*. Agric. Biol. Chem. **50**: 2061−2067.
5) Health and Welfare Statistics Association (ed), 2000. Journal of Health Welfare Statistics, Health and Welfare Statistics Association, Tokyo.
6) Hitotsubashi, S., Fujii, Y., Yamanaka, H., and Okamoto, K. 1992. Some properties of purified *Escherichia coli* heat-stable enterotoxin II. Infect. Immun. **60**: 4468−4474.
7) Hor, M., Rimpler, H., and Heinrich, M. 1995. Inhibition of intestinal chloride secretion by proanthocyanidins from *Guazuma ulmifolia*. Planta Med. **61**: 202−208.
8) Isogai, E., Isogai, H., Takeshi, K., and Nishikawa, T. 1998. Protective effect of Japanese green tea extract on gnotobiotic mice infected with an *Escherichia coli* O157:H7 strain. Microbiol. Immunol. **42**: 125−128.
9) Kanda, T., Akiyama, H., Yanagida, A., Tanabe, M., Goda, Y., Toyoda, M., Teshima, R., and Saito, Y. 1998. Inhibitory effects of apple polyphenol on induced histamine release from RBL-2H3 cells and rat mast cells. Biosci. Biotechnol. Biochem. **62**: 1284−1289.
10) Kojima, T., Akiyama, H., Sasai, M., Taniuchi, S., Goda, Y., Toyoda, M., and Kobayashi, Y. 2000. Anti-allergic effect of apple polyphenol on patients with atopic dermatitis: a pilot study. Allergol. Int. **49**: 69−73.
11) Lencer, W.I., Hirst, T.R., and Holmes, R.K. 1999. Membrane traffic and the cellular uptake of cholera toxin. Biochim. Biophys. Acta **1450**: 177−190.
12) Nalin, D.R., Cash, R.A., Islam, R., Molla, M., and Phillips, R.A. 1968. Oral maintenance therapy for cholera in adults. Lancet **ii**: 370−372.
13) Noda, M., Tsai, S.-C., Adamik, R., Bobak, D.A., Moss, J., and Vaughan, M. 1989. Activation of immobilized, biotinylated choleragen A1 protein by a 19-kilodalton guanine nucleotide-binding protein. Biochemistry **28**: 7936−7940.
14) Noda, M., Tsai, S.-C., Adamik, R., Moss, J., and Vaughan, M. 1990. Mechanism of cholera toxin activation by a guanine nucleotide-dependent 19 kDa protein. Biochim. Biophys. Acta **1034**: 195−199.
15) Ramamurthy, T., Bhattacharya, S.K., Uesaki, Y., Horigome, K., Paul, M., Sen, D., Pal, S.C., Takeda, T., Takeda, Y., and Nair, G.B. 1992. Evaluation of the bead enzyme-linked immunosorbent assay for detection of cholera toxin directly from stool specimens. J. Clin. Microbiol. **30**: 1783−1786.
16) Raufman, J.-P. 1997. Cholera. Am. J. Med. **104**: 386−394.
17) Sharpio, R.L., Kumar, L., Phillips-Howard, P., Wells, J.G., Adcock, P., Brooks, J., Ackers, M.-L., Ochieng, J.B., Mintz, E., Wahlquist, S., Waiyaki, P., and Slutsker, L. 2001. Antimicrobial-resistant bacterial diarrhea in rural western Kenya. J. Infect. Dis. **183**: 1701−1704.
18) Thorngate, J.H. 1993. Flavan-3-ols and their polymers, p. 51−63. *In* Gump, H.B., and Pruett, J.D.(eds), Beer and wine production: analysis, characterization, and technological advances. ACS Symposium Series, American Chemical Society, Washington, D.C.
19) Toda, M., Okubo, S., Ikigai, H., Suzuki, T., Suzuki, Y., Hara, Y., and Shimamura, T. 1992. The protective activity of tea catechins against experimental infection by *Vibrio choler-*

ae O1. Microbiol. Immunol. **36**: 999−1001.
20) Watten, R.H., Morgan, F.M., Songkhla, Y.N., Vanikiati, B., and Phillips, R.A. 1959. Water and electrolyte studies in cholera. J. Clin. Invest. **38**: 1879−1889.
21) World Health Organization. 2000. Cholera, 1999. Wkly. Epidemiol. Rec. **75**: 249−256.
22) Yanagida, A. 1997. Antioxidativity and other functions of apple polyphenols. Fragrance J. **4**: 63−69.