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SPECIAL REPORT

Black tea extract, thearubigin fraction, counteract the effects of botulinum neurotoxins in mice

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Botulinum neurotoxin type A (BoNT/A, 1.5 nM) completely inhibited indirectly evoked twitches in *in vitro* mouse phrenic nerve-diaphragm preparations within 40–45 min. Black tea extract, thearubigin fraction (TRB), mixed with BoNT/A blocked the inhibitory effect of the toxin. The protective effect of TRB extended to botulinum neurotoxins types B and E (BoNT/B and BoNT/E) and tetanus toxin, but not to tetrodotoxin. TRB was also effective against oral toxicity of BoNT/A, B and E. Thus, TRB may be of potential benefit in protecting the paralytic actions of botulinum neurotoxins (BoNTs), but its use is limited by mixing with the toxin.
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Abbreviations: BoNT/A, botulinum neurotoxin type A; BoNT/B, botulinum neurotoxin type B; BoNT/E, botulinum neurotoxin type E; BoNTs, botulinum neurotoxins; DT, directly induced twitches; IT, indirectly induced twitches; TRB, thearubigin fraction

Introduction Botulism is a major problem in food hygiene due to its high lethality (Kessler & Benecke, 1997). Prophylaxis, definitive diagnosis and the only effective therapy for botulism depend on chemically detoxified form(s) of the neurotoxins and the antisera raised with the toxoids (Singh & DasGupta, 1989). Agents to detoxify the effects of botulinum neurotoxins (BoNTs) have been investigated (Mikhailov & Mikhailov, 1970). Some agents were found to be effective to delay the onset of paralysis (Deshpande *et al*., 1997; Simpson, 1986). We have continued the effort to find an inactivator for BoNTs and found a convincing fraction in black tea extracts. This is the first report to describe such an inactivating substance for BoNTs in natural foodstuff.

Methods Phrenic nerve-diaphragm preparations were made from 7–9-week old *ddY* strain mice of either sex. The preparations were bathed in a modified Krebs-Ringer solution of the following composition (mM): NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 15 and glucose 11. This solution was bubbled with a mixture of 95% O_2 and 5% CO_2 and maintained at a pH of 7.3 at 36°C. A basal loading tension of 1.0 g was applied to the preparation. The nerve trunk or muscle layer of the diaphragm preparation soaked in the Krebs-Ringer solution was stimulated with supramaximal square wave pulses at 0.1 Hz with an electronic stimulator (SEN 3301; Nihon Kohden, Tokyo, Japan). Isometric force development was recorded on a thermal array recorder (AD 100F; Nihon Kohden). Thearubigin fraction (TRB) was prepared by extracting in a 1-butanol fraction from black tea leaves (Cooperative Society, Tokyo, Japan) as described by Xie *et al*. (1993). The 1-butanol fraction was evaporated and the TRB was then dissolved in 3 ml of 0.05 M sodium phosphate buffer (pH 6.0). Tannic acid (Wako, Osaka, Japan), catechin (Kurita Ind. Ltd., Tokyo), and theaflavins (Sigma, St. Louis, MO, U.S.A.) were also examined. BoNT/A (MW ≈ 500,000, Wako) was dissolved at 1 mg ml^{-1} (≈2 μM) in 0.05 M aceto-acetate-0.2 M sodium chloride buffer (pH 6.0). Tannic acid, catechin, and theaflavins were dissolved in 0.05 M sodium phosphate buffer (pH 6.0). The preparation of TRB (10 μl) was mixed with the BoNT/A samples (15 μg in 15 μl). Tannic acid and catechin, and theaflavins were mixed at 1–50 times equimolar and 1–20 times the weight ratio to the BoNT/A samples, respectively.

All procedures to the care and use of experimental animals were approved by the Animal Research Committee, Obihiro University, and they were conducted under both the Guidelines for Animal Experiment in Obihiro University and the Guiding Principles in the Use of Animals in Toxicology that were adopted by the Society of Toxicology in 1989.

Results Muscle twitches were elicited neurally (indirectly induced twitches, IT) and directly (directly induced twitches, DT). A constant amplitude was maintained for at least 3 h. The amplitude of IT was constant in the presence or absence of TRB alone (Figure 1, top panel), showing neither inhibitory nor increasing effect on the IT amplitude. The IT was abolished by BoNT/A (1.5 nM) within 40–45 min after exposure to the toxin (Figure 1, middle panel). Mixing the toxin (15 μg in 15 μl of sample) with 10 μl of TRB blocked the inhibitory effect (Figure 1, bottom panel). This protective effect was sustained for at least 3 h and was also found with BoNT/B and E (Wako) and tetanus toxin (List Biological Laboratories, Inc., CA, U.S.A.), but not with tetrodotoxin (Sankyo, Tokyo, Japan) (data not shown). Tannic acid, catechin, and theaflavins failed to block the toxic effect (Table 1).

Oral administration of BoNT/A (100 μg in 100 μl of sample $mouse^{-1}$ weighing 30–35 g) was paralytic in mice

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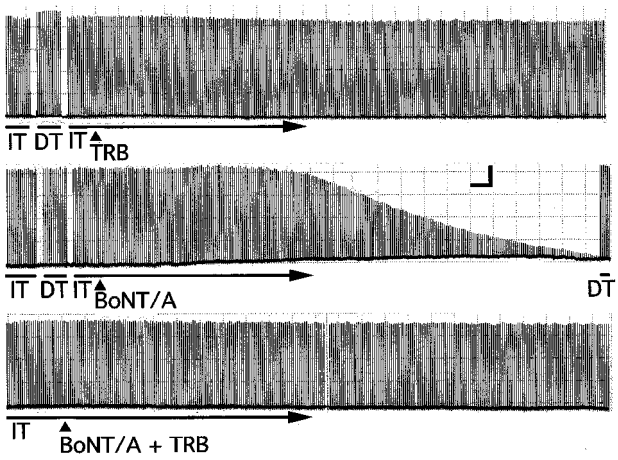

Figure 1 Representative results of the effect of thearubigin fraction (TRB) on neuromuscular blockade by botulinum neurotoxin type A (BoNT/A, 1.5 nM). IT: indirectly induced twitches, DT: directly induced twitches. Calibration: 2 min and 1 gf (gram force). All traces were derived from at least three similar observations.

Table 1 Efficacy of several tea components to protect neuromuscular blockade by BoNT/A

Samples	*Protect (+) or not (−)*
Thearubigin fraction	+
Tannic acid	−
Catechin	−
Theaflavins	−

Table 2 Protective effect of thearubigin fraction (TRB) for oral paralytic effect of BoNTs (100 μg in 100 μl mouse^{-1})

Treatments	*Number of mice tested*	*Number of mice nonparalyzed*
TRB (1)	8	8
TRB (2)	8	8
TRB (4)	12	12
BoNT/A (1)	12	0
BoNT/B (1)	8	0
BoNT/E (1)	8	0
BoNT/A (1) + TRB (1)	8	0
BoNT/A (1) + TRB (2)	8	3
BoNT/A (1) + TRB (4)	8	8
BoNT/B (1) + TRB (4)	8	8
BoNT/E (1) + TRB (4)	8	8

This dose of the toxins was taken as the dose which would induce paralysis 100% of mice within a day. The protective effect of TRB was estimated as the number of nonparalyzed mice for a 1-month observation period. (*n*), *n*-times volume ratio to BoNTs. BoNT/A, B and E, botulinum neurotoxin type A, B and E, respectively.

(Table 2). The effect of BoNT/A was also completely blocked by mixing the toxin with four times volume of the extracted TRB. The TRB was effective in blocking the effect of BoNT/B and E (Table 2) and tetanus toxin but not the effect of tetrodotoxin (data not shown), thus specific to the toxins produced by *Clostridium* family.

Discussion The present experiments demonstrated that TRB specifically blocked toxicity of BoNTs. Other candidates were examined (e.g. tannic acid, catechin, and theaflavins), but no protective effect was observed. Thearubigins are formed during fermentation by polymerization of theaflavins (Hazarika *et al*., 1984; Roberts, 1958), which were inactive in the present study. The brown acidic pigments of black tea are classified as thearubigins and the yellow, neutral pigments are classified as theaflavins (Roberts & Williams, 1958). Polyphenols can combine with proteins and perhaps polysaccharides as well (Haslam, 1974; Sanderson & Perera, 1966). Thus, TRB might inactivate the toxins by possible binding manner. Because the protective effect was maintained in oral tests, the TRB should be examined for clinical use for prophylaxis of botulism in food.

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