Black Tea Extract, Thearubigin Fraction, Counteracts the Effect of Tetanus Toxin in Mice

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The aim of this study was to find an inactivating substance for tetanus toxin in natural foodstuff. Tetanus toxin (4 μg/ml) abolished indirect twitches in *in vitro* mouse phrenic nerve-diaphragm preparations within 2.5 hr. Hot water infusion of black tea mixed with tetanus toxin blocked the inhibitory effect of the toxin. Mixing the toxin with thearubigin fraction extracted from black tea infusion produced an identical result. Furthermore, thearubigin fraction mixed with the toxin protected against the *in vivo* paralytic effect of the toxin. Thearubigin fraction had no protective effect on other toxins, such as tetrodotoxin and saxitoxin. The specific binding of [^{125}I]tetanus toxin to rat cerebrocortical synaptosomes was inhibited by mixing iodinated toxin with thearubigin fraction. These results imply that thearubigin fraction counteracts the effect of tetanus toxin by binding with toxin, and also suggest that this fraction may be able to apply for prophylaxis of tetanus.

[Exp Biol Med Vol. 226(6):577–580, 2001]

Key words: tetanus toxin; thearubigin fraction; diaphragm; synaptosome; mouse

Tetanus toxin produced by *Clostridium tetani* exhibits the strongest neurotoxicity and has caused tetanus in mammals. The toxin invaded from the wound binds to the presynaptic membrane of the neuromuscular junction, and is internalized and transported retroaxonally to the spinal cord. The spastic paralysis induced by the toxin is due to the blockade of neurotransmitter release from spinal inhibitory neurons (1). On the other hand, tetanus toxin blocks neuromuscular transmission through inhibiting the release of neurotransmitter from motor nerve terminals and thereby causes flaccid paralysis (2). Botulinum neurotoxin produced by *C. botulinum*, another clostridial neurotoxin, has a similar action and causes botulism in humans (1).

There are three classes of universal antagonists that delay or abolish the actions of all serotypes of botulinum neurotoxin and tetanus toxin: (i) lectins with affinity for sialic acid antagonize binding, (ii) drugs that block (e.g., bafilomycin) or reverse (e.g., methylamine hydrochloride) acidification of endosomes antagonize internalization, and (iii) drugs that chelate zinc antagonize intracellular expression of toxicity (3). We also have continued the effort to find an inactivator for tetanus toxin in natural foodstuff and have found a convincing fraction in black tea extracts. This is the first report to describe such an inactivating substance for tetanus toxin in natural foodstuff.

Materials and Methods

Black tea leaves (12 g) were incubated for 2 min with 90 ml of hot distilled water (100°C). The infusion was filtered with filter paper (No. 2; Whatman, Maidstone, UK) for the tea infusion sample. Sample of thearubigin fraction from the tea infusion was prepared as described by Xie *et al.* (4). After the extraction of caffeine fraction (chloroform extract) and catechins and theaflavins fraction (ethyl acetate extract), the tea infusion sample (40 ml) was extracted with 40 ml of 1-butanol. The butanol extract was dried with a rotary evaporator under vacuum. The residue (240 mg) was dissolved in 3 ml of physiological saline solution (0.15 *M* sodium chloride) for preparation of thearubigin fraction. Total phenolic content (% weight/extract weight) in thearubigin fraction was more than 80%, measured according to the method described by Singleton *et al.* (5). The value was higher than that (approximately 65%) obtained by Xie *et al.* (4).

The black tea leaves were obtained from the Cooperative Society (Tokyo, Japan). The tetanus toxin was purchased from List Biological Laboratories (Campbell, CA) and was dissolved in 0.01 *M* sodium phosphate buffer (pH 7.5). For *in vitro* experiments, the tea infusion (1–15 μl) or thearubigin fraction (80–800 μg/1–10 μl) were mixed with the tetanus toxin sample (80 μg/320 μl). For *in vivo* experiments, thearubigin fraction (4–8 mg/50–100 μl) was mixed

We thank the Hokkaido Foundation for the Promotion of Scientific and Industrial Technology for supporting this work.

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Received July 5, 2000.
Accepted February 13, 2001.

0037-9727/01/2266-0577$15.00

at 2.5 to 5 times the volume ratio of the tetanus toxin sample (2 μg/20 μl). For binding experiments, thearubigin fraction (4–32 μg/2.5–20 μl) were mixed with labeled tetanus toxin sample (45 ng/10 μl).

The phrenic nerve-diaphragm preparations were made from *ddY* strain male and female mice (30–35 g). The preparation was soaked in a modified Krebs-Ringer solution of the following composition (in millimoles): NaCl, 136; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; $NaHCO_3$, 15; and glucose, 11. The solution was bubbled with a mixture of 95% O_2 and 5% CO_2 and was 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).

Tetanus toxin and/or thearubigin fraction was administered subcutaneously within a volume of 120 μl/mouse weighing 30 to 35 g. The amount (2 μg/mouse) of tetanus toxin injected was the dose that would induce paralysis in 100% of mice within 1 day. The protective effect of thearubigin fraction was estimated as the number of nonparalyzed mice for a 1 month observation period.

Synaptosomes (P_2B fraction) were prepared using the method described by Hajós (6) from the cerebral cortices of male and female Wistar rats (200–300 g).

Tetanus toxin was iodinated according to the method described by Bakry *et al.* (7). Toxin (150 μg) in sodium borate buffer (100 mM, pH 7.9) was mixed with [^{125}I]Na (1 mCi; New England Nuclear, Boston, MA) at room temperature. The reaction was terminated at the end of 30 min by adding glycine (200 mM). [^{125}I]Tetanus toxin was separated from reactants on a Sephadex G-50 (Amersham Pharmacia Biotech, Uppsala, Sweden) column. Residual toxicity of iodinated preparation was bioassayed by the method of Kondo *et al.* (8) and 65% to 85%

[^{125}I]Tetanus toxin (0.3 nM) was mixed with synaptosomes (50 μg protein) in 1 ml of pH 7.4 buffer containing 50 mM Tris-HCl, 100 mM sodium chloride, and 1 mg/ml BSA. The binding reaction was done at 22°C for 30 min (7). The reaction was terminated by rapid filtration under vacuum through a glass-fiber filter (Whatman). Then, the filter was immediately washed three times with 5 ml of ice-cold binding buffer. Radioactivity remaining on the filter was measured directly with a gamma counter (9). Specific binding to synaptosomes was determined in parallel incubations containing a 500-fold excess of unlabeled tetanus toxin over labeled toxin.

The protein levels of synaptosomes and tetanus toxin were quantified using a kit from Bio-Rad (Richmond, CA), as described by Bradford (10).

Statistical significance of differences was assessed by Student's *t* test.

Results

Muscle twitches were elicited neurally (indirect twitches, IT) and directly (direct twitches, DT). A constant amplitude was maintained for at least 3 hr. The amplitude of the IT was constant in the presence or absence of the black tea infusion (15 μl; BTI) alone (data not shown). The IT was abolished by tetanus toxin (4 μg/ml, 80 μg/320 μl of tetanus toxin sample per 20 ml of organ bath) within 2.5 hr after exposure to the toxin (Fig. 1B). Mixing the toxin with 15 μl of BTI blocked the inhibitory effect of the toxin (data not shown). Mixing the toxin with thearubigin fraction (800 μg/10 μl) from BTI produced an identical response (Fig. 1C). The protective effect was dependent on the dose (80–800 μg/1–10 μl) of thearubigin fraction and sustained for at least 3 hr (data not shown). Thearubigin fraction (800 μg/10 μl) alone did not alter neuromuscular transmission (Fig. 1A) and the pre- or post-addition of thearubigin fraction or BTI (15 μl) into the bath medium did not block the inhibitory effect of the toxin (data not shown). Thearubigin fraction did not protect against other toxins such as tetrodotoxin and saxitoxin (data not shown).

To demonstrate that thearubigin fraction counteracts the action of tetanus toxin *in vivo,* the action of thearubigin fraction on the paralytic effect of toxin was examined in mice. Subcutaneous administration of tetanus toxin (2 μg/mouse) paralyzed 100% of the mice within 1 day. Mixing 20 μl of the toxin (2 μg) and 100 μl of thearubigin fraction (8 mg) protected against the paralytic effect of the toxin

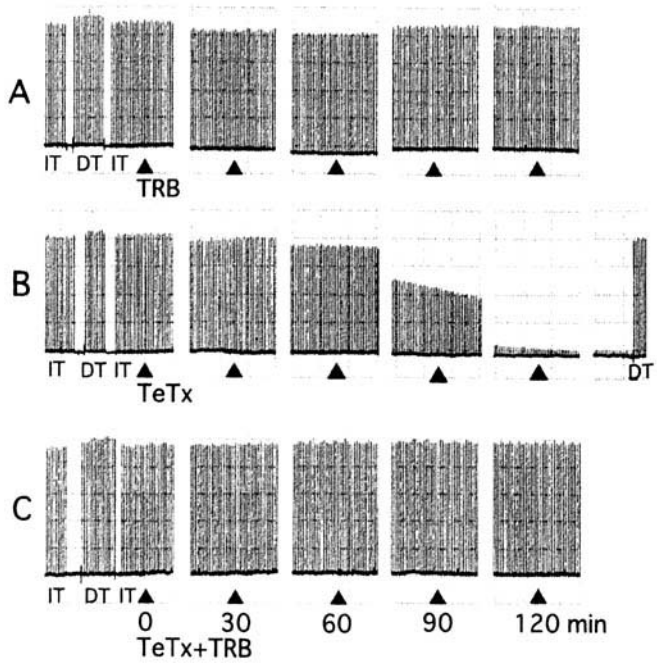

Figure 1. Representative results of the effect of thearubigin fraction (TRB) on neuromuscular blockade by tetanus toxin (TeTx, 4 μg/ml). Muscle twitches were elicited neurally (indirectly, IT) or directly (DT). TRB (800 μg/10 μl), TeTx (80 μg/320 μl) or a mixture was added into Krebs-Ringer solution (20 ml) at the 0-min arrowhead. All traces are representative of at least three similar observations. Calibration: 2 min and 1 gf (gram force).

Table I. Protective Effect of Thearubigin Fraction for Paralytic Effect of Tetanus Toxin

Treatments	Number of mice tested	Number of mice nonparalyzed
TRB (4 mg/50 μl)	9	9
TRB (6.4 mg/80 μl)	9	9
TRB (8 mg/100 μl)	9	9
TeTx	11	0
TeTx + TRB (4 mg/50 μl)	9	0
TeTx + TRB (6.4 mg/80 μl)	9	5
TeTx + TRB (8 mg/100 μl)	9	9

Note. Tetanus toxin (TeTx) and/or thearubigin fraction (TRB) were administered subcutaneously within a volume of 120 μ/mouse (30–35 g). The dose (2 μg/20 μl/mouse) of TeTx was taken as the dose that would induce paralysis 100% of mice within 1 day. The protective effect of TRB was estimated as the number of nonparalyzed mice for a 1-month observation period.

(Table I). The protective effect was dependent on the dose (4–8 mg/50–100 μl) of thearubigin fraction. Nonparalyzed mice did not show the symptom of paralysis for at least a 1-month observation period. Mixing the toxin with thearubugin fraction also delayed the onset of paralysis induced by toxin dose dependently (Fig. 2). The subcutaneous administration of thearubigin fraction at different site, or the pre- or post-administration of thearubigin fraction failed to block the paralytic effect of the toxin (data not shown).

To elucidate whether thearubigin fraction block the binding of tetanus toxin to the presynaptic membrane, the effect of thearubigin fraction on the specific binding of labeled toxin to membrane was examined using rat cerebrocortical synaptosomes. The specific binding of [^{125}I]tetanus toxin to synaptosomes was inhibited by mixing labeled toxin (45 ng/10 μl) with thearubigin fraction (32 μg/20 μl). This inhibitory effect was dependent on dose (4–32 μg/2.5–20 μl) of thearubigin fraction (Fig. 3).

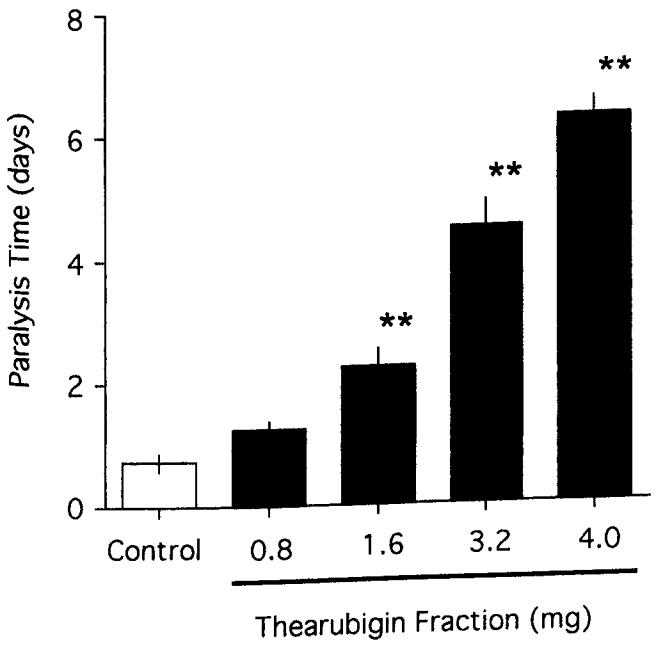

Figure 2. Effect of thearubigin fraction on the rate of onset of paralysis caused by tetanus toxin. Tetanus toxin (2 μg/20 μl/mouse) and/or thearubigin fraction were administered subcutaneously within a volume of 70 μl/mouse (30–35 g). The paralysis time was measured every 12 hr. Control indicates tetanus toxin-induced response without mixing with thearubigin fraction. Each data is the means ± SEM of values from six mice. Values significantly different from the control level are indicated: $^{**}P < 0.01$.

Discussion

The present experiments demonstrated that BTI specifically protected against toxicity from tetanus toxin *in vitro*. Some components (e.g., tannic acid, catechin, and theaflavins) in BTI were examined, but no protective effect was observed. Though the structure and chemistry of thearubigins in BTI are not yet well defined and characterized, this group has emerged as a candidate inactivator to the tetanus toxin. We extracted thearubigin fraction from BTI according to the method of Xie *et al.* (4) and tested the protective effect against the toxicity of tetanus toxin *in vitro*. Thearubigin fraction produced the same response as did BTI. Furthermore, thearubigin fraction blocked the *in vivo* paralytic effect of tetanus toxin. Therefore, it is suggested that thearubigin fraction contains an inactivating substance for tetanus toxin. This is the first report showing the protective effect of BTI against the toxicity of tetanus toxin *in vivo* and *in vitro*.

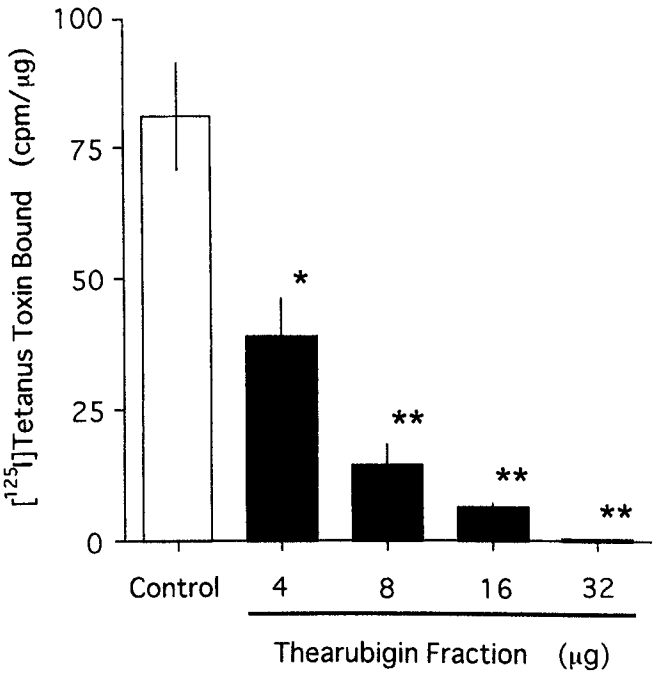

Figure 3. Effect of thearubigin fraction on the specific binding of [^{125}I]tetanus toxin to rat cerebrocortical synaptosomes. Tetanus toxin (0.3 nM) and/or thearubigin fraction was incubated with synaptosomes (50 μg/assay per ml) for 30 min at 22°C. Specific binding is the difference between total and nonspecific binding, determined in the presence of a 500-fold excess of unlabeled toxin. Control indicates the specific binding of [^{125}I]tetanus toxin without mixing with thearubigin fraction. Each data is the means ± SEM of three experiments done in duplicate. Values significantly different from the control level are indicated: $^{*}P < 0.05$, $^{**}P < 0.01$.

Thearubigins are more complex polyphenols formed during fermentation by polymerization of theaflavins (11, 12), 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 (13). Polyphenols combine with proteins and perhaps polysaccharides, as well (14, 15). Thearubigin fraction contains at least 80% of polyphenols (% weight/extract weight) and tetanus toxin is composed of protein. Furthermore, the specific binding of [^{125}I]tetanus toxin to synaptosomes was inhibited by mixing iodinated toxin with thearubigin fraction. This inhibitory effect appeared only by mixing both. Thus, it is suggested that thearubigin fraction counteracts the effect of tetanus toxin by binding with toxin.

Tetanus and botulinum neurotoxins are strikingly similar in their macrostructures (16). In addition to tetanus toxin, we demonstrated that thearubigin fraction can counteract the toxicity of botulinum neurotoxin types A, B, and E *in vitro* and *in vivo* (17). An active substance contained in thearubigin fraction has emerged as a universal inactivator of clostridial neurotoxins. This is the first substance to be identified from natural foodstuff (black tea) as a broad spectrum inactivator of clostridial neurotoxins. These findings suggest the possibility that an active substance contained in thearubigin fraction can be applied as the prophylactic drug against tetanus and food botulism by means of washing the region of injuries or adding to the foods. Additional experiments will be required to provide conclusive evidence that the observed antagonism is due to specific binding with the toxin and to elucidate the nature and the structure of the active substance present in thearubigin fraction.

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