RESEARCH ARTICLE

Clinical application of *Clostridium botulinum* type A neurotoxin purified by a simple procedure for patients with urinary incontinence caused by refractory destrusor overactivity

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Abstract

Type A neurotoxin of *Clostridium botulinum* was purified by a simple procedure using a lactose gel column. This procedure was previously reported for type B neurotoxin. Hemagglutinin-positive toxins (19S and 16S) were bound to the column under acid conditions, and the neurotoxin alone was dissociated from these hemagglutinin-positive toxins by changing the pH of the column to an alkaline condition. The toxicity of this purified toxin preparation was retained for at least 1 year at $-30\,^{\circ}C$ by supplementing it with either 0.1% albumin or 0.05% albumin plus 1% trehalose. This preparation was used to treat 18 patients with urinary incontinence caused by refractory idiopathic and neurogenic detrusor overactivity; 16 of the patients showed excellent improvement. Improvements started within 1 week after injection in most cases and lasted 3–12 months. 12S toxin dissociates into an NTX and an NTNH. Recently, we discovered that 19S toxin is a dimer of 16S toxin, and that haemagglutinin consists of four subcomponents designated AA-1, AA-2, AA-3a and HA-3b.

Introduction

Clostridium botulinum strains produce immunologically distinct neurotoxins (types A–G). The molecular masses (Mr) of types A–G neurotoxins (NTX) are *c.* 150 kDa. In culture fluid and food with acidic conditions, the NTXs associate with nontoxic components, and form large complexes called progenitor toxins (PTX). PTXs are found in three forms with Mr of 900 kDa (19S), 500 kDa (16S) and 300 kDa (12S). The 12S toxin is composed of NTX and a nontoxic component having no hemagglutinin activity (designated nontoxic nonhemagglutinin; NTNH), whereas the 19S and 16S toxins are composed of NTX, NTNH and hemagglutinin. The type A strain produces three forms of toxin (19S, 16S and 12S). Types B, C and D produce the 16S and the 12S toxins. In alkaline conditions, the PTXs dissociate into an NTX and a nontoxic component; 19S and 16S toxins dissociate into an NTX and a nontoxic component (complex) of an NTNH and a hemagglutinin, and 12S toxin dissociates into an NTX and an NTNH (Sakaguchi *et al.*, 1984; Oguma *et al.*, 1999).

Type A and B PTXs have been used to treat patients with strabismus, blepharospasm, nystagmus, facial spasm, spastic aphonia, and many other dystonias including cervical dystonia (Jankovic & Brin, 1991; Lew *et al.*, 2000). In both toxin types, PTXs are used because they are easily obtained and are

more stable than NTX. The treatment is very effective, but a serious problem is that anti-PTX, including anti-NTX antibodies, is produced after several injections in some patients. We think that using NTX alone may be better than using the PTX because both HA1 and HA3b have immunoenhancing activity, although there are no definite data in humans (Lee *et al.*, 2005). Recently, we established a simple new procedure for the large-scale purification of botulinum type B PTX and NTX, which have fully activated toxicity, by employing a lactose gel column (Arimitsu *et al.*, 2003). In this study, we report that type A NTX can also be purified by a similar procedure as type B, and can be stocked for a long period at $-30\,^{\circ}C$ without reducing its toxicity if albumin and trehalose are added to the preparation. Furthermore, the effects of injecting of this toxin preparation into the bladder muscle of the patients with urinary incontinence caused by idiopathic and neurogenic detrusor overactivity refractory to antimuscarinic drugs are described.

Materials and methods

Bacterial strain and culture for toxin production

Clostridium botulinum type A, strain 62A, was used. The cells were cultured at 35 °C overnight in 50 mL of cooked meat medium (pH 7.2; Difco Laboratories, Detroit, MI) supplemented with 1% proteose peptone (Difco Laboratories), 1% polypeptone (Nihon Pharmaceutical Co., Ltd, Osaka, Japan), 1% lactalbumin hydrolysate (Difco Laboratories), 1% yeast extract (Difco Laboratories), and 0.1% L-cysteine hydrochloride monohydrate. The medium for toxin production was composed of 2% peptone (Sigma, Japan), 0.5% yeast extract (Difco Laboratories), 0.5% glucose and 0.025% sodium thioglycolate. Before autoclaving, it was adjusted to pH 7.2 with a 10% NaOH solution. A 10 mL sample of the culture from the 50 mL of cooked meat medium was inoculated into 4000 mL of medium, which was incubated at 30 °C for 5 days. For every batch of toxin purification, four flasks were used.

Partial purification of toxin

A whole culture (16 000 mL) was adjusted to pH 4.0 with 3 N H_2SO_4 and kept standing overnight at room temperature to allow the precipitate to settle (Sugii & Sakaguchi, 1975). The supernatant fluid was removed by siphoning. About four volumes of distilled water was added to the remaining precipitate to dissolve acid-soluble materials, and the mixture was allowed to stand overnight at room temperature. The supernatant fluid was again removed by siphoning. The precipitate was packed by centrifugation at 8700 ***g*** for 20 min at 4 °C, suspended in 800 mL of 0.2 M phosphate buffer (pH 6.0). After being adjusted to pH 6.0 with a 10% NaOH solution, it was kept standing for 1 h at 37 °C with occasionally stirring, and stored overnight at 4 °C. The precipitates which appeared were removed by centrifugation two times at 8700 ***g*** for 20 min at 4 °C, and then a 2% protamine solution of 0.2 M phosphate buffer (pH 6.0) was added to the supernatant to precipitate RNAs. After centrifugation at 8700 ***g*** for 20 min at 4 °C, the toxins were then precipitated from the supernatant with a 60% saturation of ammonium sulfate, collected by centrifugation at 8700 ***g*** for 20 min at 4 °C, dissolved in 100 mL of 50 mM sodium acetate buffer (pH 4.2), and then dialyzed overnight against the same buffer. The precipitates which appeared during dialysis were removed by centrifugation at 15 900 ***g*** for 20 min. From the resultant supernatant (crude toxin preparation), the PTXs and NTX were purified by the procedure using SP-Sepharose and β-lactose gel columns that was developed for purifying type B toxins (Arimitsu *et al.*, 2003).

Purification of toxin

All the chromatography steps were performed at room temperature, and the dialysis steps were performed at 4 °C. The crude toxin preparation (100 mL) was first applied to a column (1.4 cm × 26 cm) of SP-Sepharose (Pharmacia Biotechnology AB, Uppsala, Sweden) equilibrated with 50 mM sodium acetate buffer. The proteins were eluted with a NaCl gradient (0–0.5 M), and 2.3 mL fractions were collected. Four protein peaks (peaks 1–4) were eluted (Fig. 1). The fractions of peak 3, containing hemagglutinin-positive 19S and 16S toxins, and peak 4, containing hemagglutinin-negative 12S toxin, were pooled (77 mg per 64 mL) and dialyzed against 10 mM sodium phosphate buffer (pH 6.0). The preparation was divided into four, and each part (16 mL) was applied to an aminophenyl β-lactose gel column (1.0 cm × 6.0 cm; E-Y Laboratories Inc., San Mateo, CA) equilibrated with the same buffer. The flow-through fractions were 12S toxin-rich, whereas the fractions eluted by the same buffer containing 0.05 M lactose were hemagglutinin-positive PTXs (19S and 16S). For purification of the NTX, the purified 19S and 16S toxin fractions (28–30; 10.7 mg per 7 mL) thus obtained were first dialyzed against 10 mM sodium phosphate buffer (pH 8.0) to dissociate them to an NTX and nontoxic components, and then applied to the lactose gel column equilibrated with the same buffer (Fig. 2b). The NTX passed through the column, whereas the nontoxic components bound to the column (the latter was eluted by the same buffer containing 0.05 M lactose).

Determination of toxicity and hemagglutinin titers

The preparations were diluted in serial 10-fold steps with 20 mM sodium phosphate buffer (pH 6.0) containing 0.2% (w/v) gelatin, and 0.5 mL of each dilution was

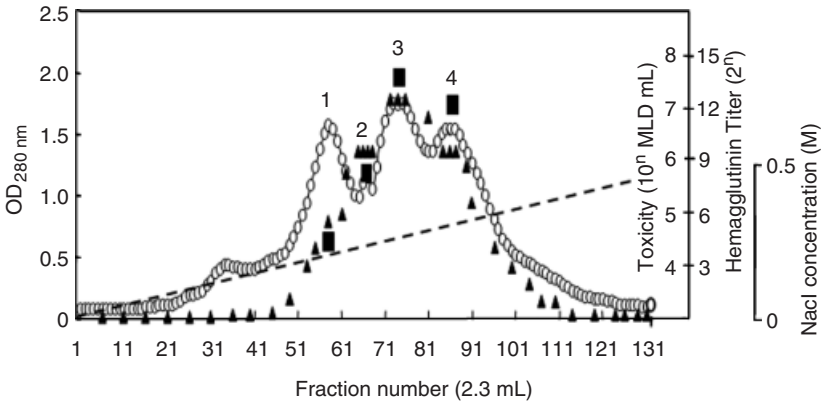

Fig. 1. SP-Sepharose fast flow chromatography. The ammonium sulfate fraction from the culture fluid (crude toxin preparation) was applied to a column equilibrated with a 50 mM sodium acetate buffer (pH 4.2). Elution was performed by increasing the concentration of NaCl to 0.5 M, and 2.3 mL fractions were collected. O, OD280; ▲, hemagglutinin titer; ■, toxicity. The dashed line represents the concentration of NaCl.

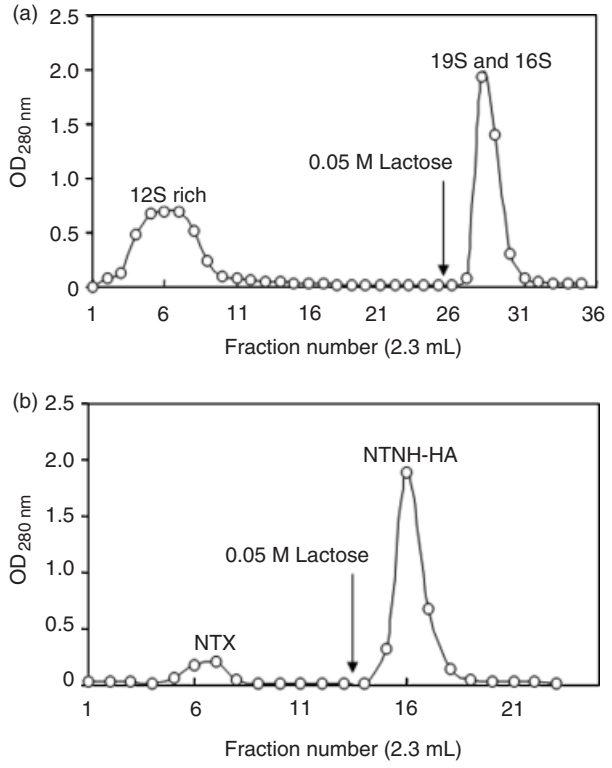

Fig. 2. (a) Separation of 12S-rich and 19S and 16S toxins by beta-lactose gel affinity column chromatography. Fractions 69–96 in Fig. 1 were pooled (19S and 16S toxin fraction; 77 mg per 64 mL), and its 1/4 volume (19 mg per 16 mL) was applied to the column at pH 6.0. O, $OD_{280\ nm}$. (b) Separation of the NTX and NTNH-HA by β-lactose gel affinity column chromatography from the 19S and 16S toxin. The purified 19S and 16S toxin (10.7 mg per 7 mL) dialyzed against the buffer (pH 8.0) was applied to the column equilibrated with the same buffer. After the unbound protein was collected, the bound protein was eluted with the same buffer containing 0.05 M lactose. O, $OD_{280\ nm}$.

intraperitoneally injected into three white mice (ddy, female, about 20 g). The mice were observed for 1 week, and the minimal lethal dose (MLD) per milliliter was measured. The hemagglutinin titer was obtained with microtitration in multiwell plates. The toxin solution was diluted in serial twofold steps with 10 mM phosphate-buffered saline (PBS) (pH 7.4) and mixed with an equal volume of 1% suspension of washed human erythrocytes (group O). After incubation at room temperature for 2 h, the reciprocal of the highest dilution at which hemagglutination was positive was denoted as the hemagglutinin titer (2^n).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a 12.5% separating gel, and Mr was determined using Perfect Protein Markers (6.5–200 kDa; Bio-Rad Lab., Hercules, CA). Protein bands were visualized by staining with Coomassie brilliant blue (CBB) R-250 (Bio-Rad).

Stability of toxicity of the NTX

The NTX was filtered with a 0.22 μm pore size membrane filter and then diluted to 1000 MLD mL^{-1} in Isotonic Sodium Chloride Solution (Otsuka Pharmaceutical Co., Ltd) with or without human serum albumin (1, 2.5, 5, 10, 50 mg mL^{-1}). After storage of these preparations (0.5-mL volume each, in vials) at 4, −30 or −80 °C for 1, 3, 6 or 12 months, the level of remaining toxicity was determined in an assay using mice (Table 1). Thereafter, the effects of combinations of human serum albumin (0.5 mg mL^{-1}), trehalose (1%) and Tween-20 (0.01%) on toxicity were studied by storing the preparation for long periods at −30 °C (Table 2).

Cytotoxicity on different cell lines

IMR-32 (Human, Abdominal mass), Neuro-2a (Mouse, Spinal cord region), U937 (Human, Pleural effusion), NOMO-1 (Human, Peripheral blood), HT-29 (Human, Colon), Caco-2 (Human, Colon), Hela (Human, Cervix) and Vero (African green monkey, Kidney) cells were used. The cells were grown in Dulbecco's Modified Eagle's

Table 1. Stability of NTX under different storage conditions*

Storage temperature	Albumin concentration	Toxin titer (MLD per 0.5 mL) Storage period (months) 1	3	6	12
4 °C	Free	100	< 50	ND	ND
	0.1% (1 mg mL^{-1})	400	50	50	< 50
	0.25% (2.5 mg mL^{-1})	400	50	50	50
	0.5% (5 mg mL^{-1})	400	400	200	100
	1% (10 mg mL^{-1})	500	500	500	200
	5% (50 mg mL^{-1})	500	500	500	200
− 30 °C	Free	200	100	50	< 50
	0.1% (1 mg mL^{-1})	500	500	500	500
	0.25% (2.5 mg mL^{-1})	500	500	500	500
	0.5% (5 mg mL^{-1})	500	500	500	500
	1% (10 mg mL^{-1})	500	500	500	500
	5% (50 mg mL^{-1})	500	500	500	500
− 80 °C	Free	200	100	50	50
	0.1% (1 mg mL^{-1})	500	500	500	500
	0.25% (2.5 mg mL^{-1})	500	500	500	500
	0.5% (5 mg mL^{-1})	500	500	500	500
	1% (10 mg mL^{-1})	500	500	500	500
	5% (50 mg mL^{-1})	500	500	500	500

*The NTX was diluted to 500 MLD per 0.5 mL in buffer with or without human albumin, and stored at different temperatures. After storage, the toxin titer remaining was determined.
ND, not determined.

Table 2. Stability of NTX under storage with or without human albumin, trehalose, and Tween-20*

Storage temperature	Sample mixtures	Toxin titer (MLD per 0.5 mL) Storage period (months) 1	3	6	12
− 30 °C	NTX+Alb+Tre+T-20	500	500	500	500
	NTX+Alb+Tre	500	500	500	500
	NTX+Alb+T-20	500	400	400	100
	NTX+Tre+T-20	500	200	200	< 50
	NTX+T-20	400	100	50	< 50
	NTX+Alb	500	400	400	100
	NTX+Tre	400	200	50	< 50

*The NTX was diluted to 500 MLD per 0.5 mL in buffer with or without human albumin (0.05%), trehalose (1%), and Tween-20 (0.01%). After storage, the toxin titer remaining was determined.

Medium (DMEM) or RPMI 1640 medium (Gibco) supplemented with 10% (v/v) fetal calf serum at 37 °C in 5% CO_2 incubator. The grown cells were adjusted to 1×10^6 cells mL^{-1}. The cell suspension, 1 mL, was distributed into each well of 24-well tissue culture plates (Cellstar), followed by the addition of 0.1 mL of human albumin (50 mg mL^{-1}), type A NTX (1000 or 10 000 MLD mL^{-1}) or only isotonic sodium chloride solution as a control. After incubation for 3–4 days at 37 °C in a 5% CO_2 incubator, the cells were analyzed by microscopy (IX71, Olympus Optical Co. Ltd, Tokyo, Japan). In the cases of IMR-32, Neuro-2a and U937 cells, the cytotoxicity was also investigated using a Cyto Tox 96 Non-Radioactive Cytotoxicity Assay (Promega). The cells (1×10^5 cells per 0.1 mL) were distributed into each well of 96-well tissue culture plates (Cellstar), followed by the addition of 0.1 mL of DMEM containing 20 μL of type A NTX (10 000 MLD mL^{-1}) or human albumin (50 mg mL^{-1}). Three wells were prepared for each preparation. After incubation for 4 h at 37 °C in a 5% CO_2 incubator, the supernatant, 0.1 mL, of each well was transferred to fresh 96-well flat-bottom (enzymatic assay) plates, and then 50 μL of substrate mix was added. The plates were

covered with aluminum foil for protection from light, and kept for 30 min at room temperature. The reaction was stopped with 50 μL of stop solution, and the color was read by a microplate reader (Bio-Rad) using a 490 nm wavelength. As positive and negative controls, 9% Triton X-100 and Isotonic Sodium Chloride Solution were added into the cells respectively ($n = 3$). The cytotoxicity level was statistically analyzed using Scheffe's multiple comparison test and differences were considered significant at P values < 0.05. Results were expressed as means ± SD.

AMES test

The mutagenicity assay of the NTX (1000 or 10 000 MLD mL^{-1}) and albumin (50 mg mL^{-1}) was performed according to the preincubation technique of Yahagi *et al.* (1977). The tester strain was *Salmonella typhimurium* YG1024, which detects frameshift-type mutagens. The assays were done in the presence of an S9 mix. The S9 mix was prepared from the livers of Sprague–Dawley rats that had been treated with polychlorinated biphenyl (KC-54, chlorine content 54%). NTX or albumin were dissolved in 0.1 mL dimethylsulfoxide and used for the assay. Judgement for a positive mutagenicity was given to results producing more than threefold the number of revertants over the average of solvent (dimethylsulfoxide)-only controls (Kira *et al.*, 1995).

Prionics check with Western blot

Contamination of the NTX preparations (300 μg mL^{-1}), hemagglutinin-positive PTX (19S and 16S; 0.3, 1, 3 mg mL^{-1}), and albumin (0.5, 5 mg mL^{-1}) with prion protein was assayed using a Prionics-Check Western kit (Prionics AG). Gel electrophoresis was performed on a 12% NuPAGE gel (Invitrogen) using an Xcell Sure Lock Mini-Cell (Invitrogen). A 10 μL aliquot of the control positive sample containing prion or the above samples, which had been boiled for 7 min, was loaded in the lanes of the gel, and then the inner and outer electrophoresis chambers were filled up with NuPAGE SDS/MOPS running buffer (Invitrogen). Antioxidant (500 μL) was then added into the inner chamber only. The proteins from the gel electrophoresis were electroblotted onto polyvinylidene difluoride membranes (Millipore) with a TE22 Mini Tank Transphor Unit (Amersham Bioscience) at 4 °C. After blocking with PVDF blocking buffer for 30 min at room temperature and washing with Tris-buffered saline (TBS)-Tween, the membrane was reacted for 1 h at room temperature with 1 : 5000 diluted Prion-Specific mouse monoclonal antibody 6H4 (Prionics AG) in PVDF blocking buffer. This was washed again, and then reacted with 1 : 5000 diluted secondary antibody (goat anti-mouse IgG coupled with alkaline phosphatase) (Prionics AG) for 30 min at room temperature. After washing with TBS-Tween, the chemiluminescence signal was generated by reaction on X-ray film (Hyperfilm, Amersham Bioscience) with CDP-Star (Lumi-Imager System, Roche Diagnostics GmbH).

Endotoxin check

The NTX was filtered with a 0.22 μm pore size membrane filter and then diluted to 100 000 MLD mL^{-1} in isotonic sodium chloride solution. Contamination with endotoxin was determined in a Limulus amoebocyte lysate assay (BioWhittaker) (Haan *et al.*, 2002; Gao & Tsan, 2003).

Treatment with NTX preparation

Since we obtained the permission from our university ethics committee for clinical trials of this NTX preparation, we have started treating patients with urinary incontinence caused by idiopathic and neurogenic destrusor overactivity refractory to antimuscarinic drugs. After obtaining informed consent, a total 50–200 MLD (or units, U) of NTX was injected into the destrusor of patients through rigid or flexible cystoscopy. The NTX was suspended in 10–20 mL of Isotonic Sodium Chloride Solution to a final concentration of 50–200 U in which several drops of indigocarmine (4 mg mL^{-1}, Daiich Pharmaceutical Co. Ltd) was added, and injected in 20–40 points, each *c.* 0.5 mL at an equivalent of 2.5–10 U per injection, of the destrusor, including the trigone. As an antibiotic prophylaxis, all patients received 300 mg levofloxacin orally for 2 days. All patients were followed up at 7, 30 and 90 days after treatment. Filling cystometry was used to evaluate bladder function before and 30 days after NTX treatment. Moreover, patients were also asked to complete a 3-day bladder diary and a validated quality of life survey (I-QOL, visual analog scale) before treatment, and 7, 30 and 90 days after treatment.

Cystometry

Urodynamic investigation was performed on all patients with the same unit (Laborie Medical Technologies UDS 5100). Intravesical pressures were determined with transurethral catheters placed under strictly sterile conditions. Eight-Fr double lumen catheters were used. Rectal pressures were determined using a waterline system consisting of a transrectal balloon catheter filled with 10 mL water. During cystometry the bladder was filled with physiological saline at 50 mL min^{-1}. The methods of UDS were in accordance with recommendations from the International Continence Society (Griffiths *et al.*, 1999).

QOL score

Patients scored their present continence condition on a 10-point analog scale (0 = continence, 10 = complete

incontinence). The I-QOL data were scored, with a range of possible scores from 22 to 110 (representing 22 questions with answers scored between 1 and 5) (Wagner *et al.*, 1996). The conversion used a formula putting the total scores on a 0–100 scale, which were then rounded to one decimal place. When patients became dry or had a decrease in incontinence episodes of more than 50%, they were considered to have had symptom improvement.

Results and discussion

Purification of PTXs

The cells were incubated in a 5 L flask as described in 'Materials and methods'. For type B, the cells were incubated in a cellophane tube. Previously, for purification of type A, we used the same cellophane tube procedure as type B (Inoue *et al.*, 1996). This time, however, we found that the toxin amount produced by the procedure using a flask was much higher than the cellophane tube procedure in type A. The PTXs were precipitated with acid and ammonium sulfate, and then purified by column chromatography, similar to type B. On a SP-Sepharose Fast Flow column, four protein peaks (peaks 1–4) were eluted by the NaCl gradient (Fig. 1). The toxic activity of peak 1 and peak 2 was low (10^{4-5} MLD mL^{-1}), whereas the toxicity of peaks 3 and 4 was high ($\sim 10^7$ MLD mL^{-1}). The hemagglutinin activity of peaks 1, 2, 3, and 4 was 2^5, 2^{10}, 2^{12}, and 2^{11}, respectively. Although these four peaks were not well separated, it was postulated that, based on the previous report (Inoue *et al.*, 1996) and SDS-PAGE banding profile of each peak (Fig. 4a), peak 3 contains mainly hemagglutinin-positive PTXs (19S and 16S), and peak 4 contains a hemagglutinin-negative PTX (12S), and that peak 1 and peak 2 are HA1 and hemagglutinin, respectively, which exist freely in the culture. The fractions of peaks 3 and 4 were pooled (77 mg per 64 mL; hemagglutinin activity, 2^{10}; toxic activity, 2.0×10^7 MLD mL^{-1}), dialyzed against a 10 mM sodium phosphate buffer (pH 6.0), and then applied to an aminophenyl β-lactose gel column equilibrated with the same buffer. The flow-through fractions showed high toxicity with quite low hemagglutinin activity (hemagglutinin, 2^1; toxic activity, 1.6×10^6 MLD mL^{-1}), whereas the fractions eluted by the same buffer containing 0.05 M lactose showed both a high hemagglutinin titer and high toxicity (hemagglutinin, 2^{12}; toxic activity, 4.0×10^7 MLD mL^{-1}), indicating that the former is a 12S-rich toxin and the latter has hemagglutinin-positive 19S and 16S toxins (Fig. 2a). For purification of the NTX, the purified 19S and 16S toxin fractions (28–30; 10.7 mg per 7 mL) thus obtained were first dialyzed against a 10-mM sodium phosphate buffer (pH 8.0), and then applied to a lactose gel column equilibrated with the same buffer (Fig. 2b). The NTX passed through the column (hemagglutinin activity, 2^0; toxic activity, 4.0×10^6 MLD mL^{-1}, total 9.2 mL), whereas the nontoxic component bound to the column, and it (2^{11} of hemagglutinin and 1.6×10^5 MLD mL^{-1} of toxicity) was eluted by the same buffer containing 0.05 M lactose.

NTX could be obtained more simply as follows. After 16 mL of pooled preparation of peak 3 and peak 4 in 10 mM sodium phosphate buffer (pH 6.0) was applied to a lactose gel column equilibrated with the same buffer (the hemagglutinin-positive toxins bind to the column as mentioned above), the pH of the column was then changed with 100 mM sodium phosphate buffer (pH 8.0). Due to this change of pH, the NTX dissociated from the nontoxic components on the column; 11.5 mL of NTX fraction showing 4.0×10^6 MLD mL^{-1} of toxicity, but no hemagglutinin activity, was obtained. A nontoxic component-rich preparation (hemagglutinin activity; 2^{11}, toxic activity; 1.6×10^5 MLD mL^{-1}) was then eluted with a 10-mM sodium phosphate buffer (pH 6.0) containing 0.05 M lactose (Fig. 3).

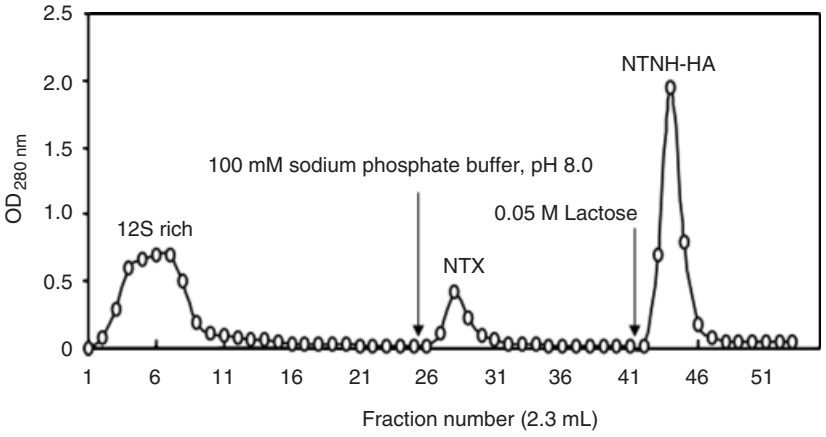

Fig. 3. Simple procedure for purifying NTX by β-lactose gel affinity column chromatography. The 19S and 16S preparation (19 mg per 16 mL) was applied to the column at pH 6.0 (10 mM sodium phosphate buffer). The pH of the column was then changed with a 100 mM sodium phosphate buffer (pH 8.0). Due to this pH change, the NTX dissociated from the nontoxic components on the column. O, $OD_{280\,nm}$.

Purity of the preparations

The three preparations – (1) hemagglutinin-positive toxin (mixture of 19S and 16S), (2) NTX, and (3) a complex of NTNH and hemagglutinin (NTNH-HA) – purified by a lactose gel column were analyzed by SDS-PAGE (Fig. 4b). As expected (Inoue *et al.*, 1996; Oguma *et al.*, 1999), the NTX demonstrated only two bands of H (~100 kDa) and L (~50 kDa) (lane 1), the NTNH-HA preparation demonstrated one NTNH band (~120 kDa) and four hemagglutinin bands (HA1, HA2, HA3a, HA3b). HA3a consisted of several bands with a similar MW) (lane 2), and the hemagglutinin-positive toxin demonstrated seven bands; H and L of NTX, NTNH, and four bands of hemagglutinin (lane 3). The NTX and NTNH-HA preparations purified by the simpler procedure also demonstrated the same banding profiles (data not shown).

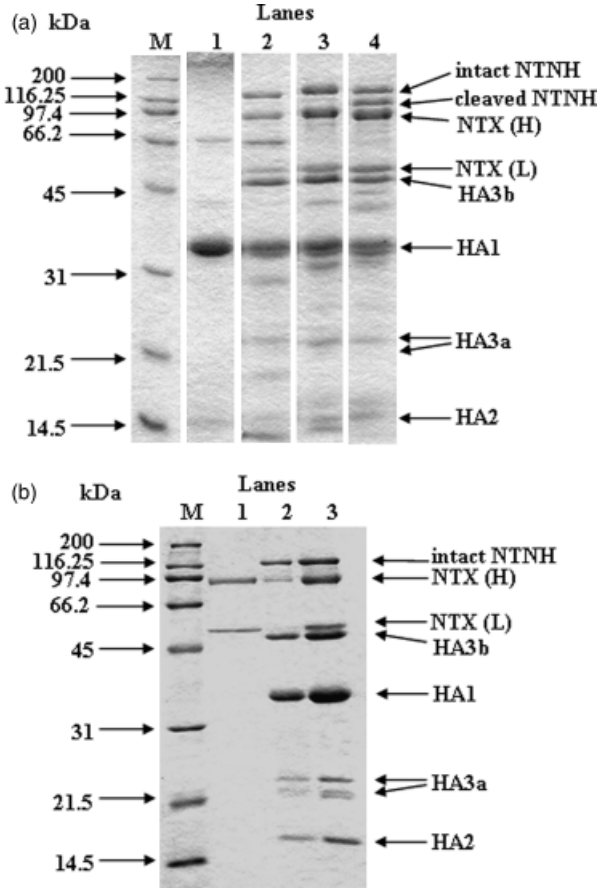

Fig. 4. SDS-PAGE analysis. (a) Each 8 μL of nondiluted main fraction of four peaks from a SP-Sepharose column, fraction No. 58 in peak 1 (lane 1); No. 67 in peak 2 (lane 2); No. 74 in peak 3 (lane 3); No. 86 in peak 4 (lane 4), and (b) three purified proteins by a lactose gel column, 3.7 μg of NTX (lane 1); 12.6 μg of NTNH-HA (lane 2); 15.5 μg of mixture of 19S and 16S (lane 3) were analyzed by SDS-PAGE. Molecular mass markers (in kilodaltons) are on the left of the gel (M).

Stability of toxicity of the NTX

In type B, no reduction of toxicity was observed when the NTX preparation was supplemented with 0.05% human albumin and stocked at 4 °C or −80 °C for 180 days (Arimitsu *et al.*, 2003). On the contrary, the toxicity of type A was decreased even though 0.05% albumin was added. Therefore, we first checked what percentage of albumin is necessary to maintain the toxicity. Preparations containing 0.1, 0.25, 0.5, 1 and 5% of albumin were prepared, and stocked at 4, −30 or −80 °C for 1 year. The toxicity was decreased at 4 °C even though 5% of albumin was added. However, under storage at −30 °C and −80 °C, no reduction was observed if more than 0.1% albumin was supplemented (Table 1). The effects of albumin (0.05%), trehalose (1%) and Tween 20 (0.01%) on the toxicity of the preparations that remained in storage at −30 °C were observed (Table 2). The toxicity was fully maintained in the cases where both albumin and trehalose were added.

We are now planning to investigate the effects of addition of another sugar or of keeping the preparations in freeze-dry.

Quality check of the NTX preparation

Cytotoxicity test with different cell lines and AMES test

The cytotoxic effects of NTX (1000 or 10 000 MLD mL^{-1}) or albumin (50 mg mL^{-1}) on IMR-32, Neuro-2a, U937, NOMO-1, HT-29, Caco-2, Hela and Vero cells in 24-well tissue plates were observed. No morphological change was observed in any cell lines when either albumin or NTX was added (Fig. 5a–d). Both NTX (10 000 MLD mL^{-1}) and albumin (50 mg mL^{-1}) again showed no cytotoxicity on IMR-32, Neuro-2a or U937 cells in the Cyto Tox 96 Non-Radioactive Cytotoxicity Assay test (Fig. 5e). In these preparations, NTX (1000 or 10 000 MLD mL^{-1}) and albumin (50 mg mL^{-1}) also showed no positive reaction on an AMES test.

Contamination check with prion, viruses and endotoxin

Contamination of the preparation with prion, AIDS virus or human hepatitis virus (types B and C) was checked. The prion protein was checked by us using Prionics-Check Western kit, and AIDS and hepatitis viruses were assayed with PCR by a commercial company (SRL, Tachikawa, Tokyo). To increase the thoroughness of the check, not only the NTX preparation but also the hemagglutinin-positive toxin preparation (19S and 16S) and albumin with different amounts (10–100 times higher amounts than employed for the treatment) were checked. In the prion check, the

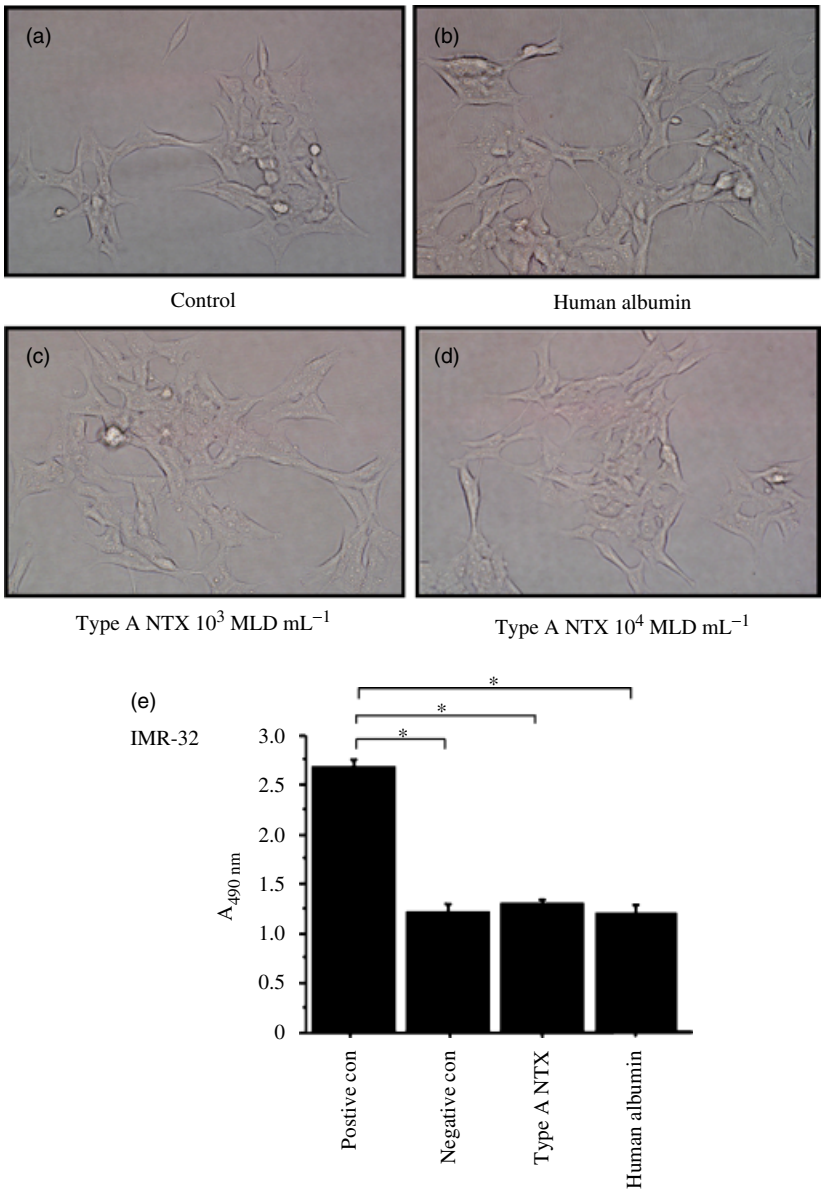

Fig. 5. Cytotoxicity test with IMR-32 cells. The results of cytotoxicity tests with albumin (50 mg mL^{-1}) and NTX (1000 or 10 000 MLD mL^{-1}) against IMR-32 cells are shown. The cells (1 mL) in 24-well tissue culture plates were treated with 0.1 mL of albumin or NTX, and then observed by microscopy (a–d). Also, the cells (0.1 mL) in 96-well tissue culture plates were treated with 20 μL of albumin or NTX, and investigated by Cyto Tox 96 Non-Radioactive Cytotoxicity Assay (e). *Significant difference ($P < 0.01$).

positive-control sample containing the isoform of a normal prion protein demonstrated a wide (not uniform) band with 25–35 kDa caused by the difference of the saccharification pattern (Fig. 6, lane 1). The preparations of 19S and 16S, NTX and albumin demonstrated no such a band. In PCR for AIDS and hepatitis, no positive products were identified. In addition, contamination with endotoxin was checked by a Limulus amoebocyte lysate assay. All the preparations were negative in this test, too.

Clinical trial

A total of 50–200 U of NTX was injected through rigid or flexible cystoscopy into the destrusor of patients who had been suffering from urinary incontinence caused by idiopathic and neurogenic destrusor overactivity refractory to antimuscarinic drugs. Thirteen men and five women, aged 20–80 years old (median 58) were treated. Ten patients were idiopathic and eight patients had neurogenic detrusor overactivity due to spinal cord injury (Table 3). When we did the first trial, we could not confirm whether the injection was complete or not. Therefore, we added several drops of indigocarmine (4 mg mL^{-1}), which is not harmful to humans, to confirm the injection without leakage.

Sixteen of the 18 patients noted improvement and nine of them became dry. Two patients noticed no change in the degree of incontinence and frequency after treatment. The mean maximal cystometric capacity increased from

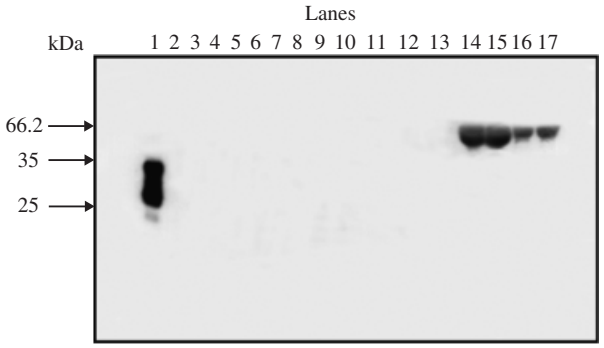

Fig. 6. Prion protein check by Western blot. Contamination of type A 19S and 16S, NTX, and human albumin with prion was checked by Prionics-Check Western kit. Lanes: 1, control (prion protein); 2, 3, and 4, 19S and 16S (3 mg mL^{-1}); 5, 6, and 7, 19S and 16S (1 mg mL^{-1}); 8, 9, and 10, 19S and 16S (300 μg mL^{-1}); 11, 12, and 13, NTX (300 μg mL^{-1}); 14 and 15, albumin (5 mg mL^{-1}); 16 and 17, albumin (500 μ mL^{-1}).

Table 3. Clinical characteristics of patients and the change of cystometric parameters before and after treatment

	Idiopathic destrusor overactivity	Neurogenic destrusor overactivity
n	10	8
Age (median)	52–80 (68)	20–61 (33)
Male : Female	5 : 5	8 : 0
Maximum cystometric capacity (mL)		
Before	142 ± 10.2	
After	320 ± 33.9*	
Maximum detrusor Pressure at the detrusor contraction (cmH_2O)		
Before	72.4 ± 8.4	
After	31.9 ± 4.6*	

*$P < 0.05$ as compared with pretreatment.

142 ± 10.2 mL at baseline to 320 ± 33.9 mL at 30 days ($P = 0.0005$). The mean maximal detrusor pressure at the detrusor contraction decreased from 72.4 ± 8.4 mmHg at baseline to 31.9 ± 4.6 mmHg at 30 days ($P = 0.001$) (Table 3). The mean number of episodes of urinary incontinence daily decreased from 5.2 ± 0.9 to 1.8 ± 0.6 ($P = 0.0004$) at 7 days, to 1.6 ± 0.7 ($P = 0.0009$) at 30 days, and to 2.2 ± 0.5 ($P = 0.005$) at 90 days. The mean urinary frequency daily decreased from 11.7 ± 1.0 to 9.9 ± 0.9 ($P = 0.0049$) at 7 days, to 9.1 ± 0.9 ($P = 0.0033$) at 30 days, and to 8.6 ± 0.9 ($P = 0.0021$) at 90 days (Fig. 7). The mean score of I-QOL at baseline improved from 61.1 ± 4.0 to 72.7 ± 4.5 ($P = 0.0043$) at 7 days, to 80.5 ± 4.1 ($P = 0.0075$) at 30 days, and to 74.3 ± 4.6 ($P = 0.007$) at 90 days. VAS score also significantly improved from 7.8 ± 0.5 to 3.3 ± 0.7 ($P = 0.007$) at 7 days, to 2.2 ± 0.7 ($P = 0.0006$) at 30 days, and to 4.1 ± 0.9 ($P = 0.0075$) at 90 days (Fig. 8). In the 16 patients who showed significant relief with decreased incontinence at the initial 1-month follow-up, the clinical

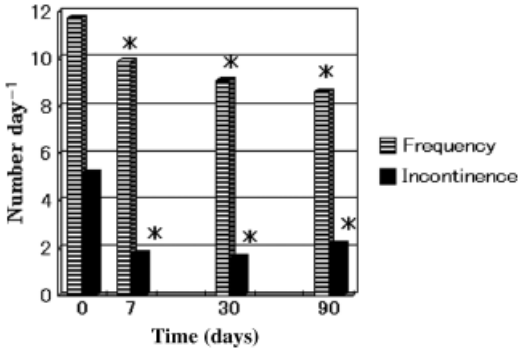

Fig. 7. Mean daily frequency and number of episodes of urinary incontinence at baseline, and 7, 30, and 90 days after treatment. **$P < 0.05$.

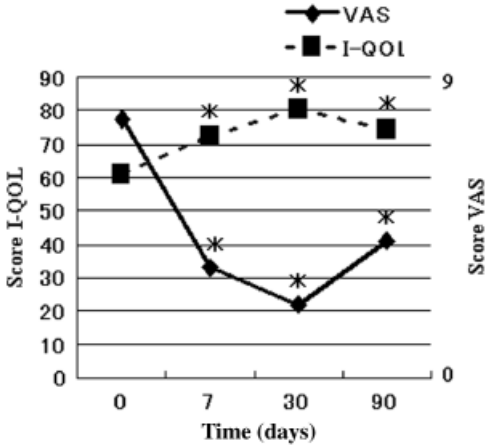

Fig. 8. I-QOL and VAS score at baseline, and 7, 30, and 90 days after treatment. Values are expressed as mean plus or minus SE. **$P < 0.05$.

improvement lasted from 3 to 12 months, with a median of 6 months. As for side effects, incomplete urinary retention occurred in only one patient with idiopathic destrusor overactivity. This patient became well by treatment with clean intermittent self catheterization for 12 months.

Dykstra *et al.* (1988) first evaluated the efficacy of type A botulinum toxin injection in treating spinal cord injured patients suffering from detrusor external sphincter dyssynergia. Thereafter, Schurch *et al.* (2000) found that the toxin can also be used for spinal cord injured patients with incontinence resistant to anticholinergic medication. They injected a total of 200–300 U of botulinum type A toxin into 20–30 sites of detrusor muscle of 31 patients, and demonstrated that mean reflex volume, mean maximum bladder capacity, and mean maximum detrusor voiding pressure were significantly recovered by injection with *c.* 300 U of toxin. Moreover, they reported the efficacy and safety of either 200 or 300 U of type A toxin injection therapy for a total of 59 patients with urinary incontinence caused by neruogenic detrusor overactivity in a randomized placebo controlled study (Schurch *et al.*, 2005). Since then, type A

toxin injections have extended beyond the realm of neurogenic bladders to patients with nonneurogenic voiding and storage disorders (Radziszewski *et al.*, 2001). Recently, Schmid *et al.* (2006) reported that 88% of 100 cases of idiopathic detrusor overactivity showed significant improvement by the injection with 100 U of type A toxin, and that mean efficacy duration was at least *c.* 6 months. Smith *et al.* (2005) also reported that maximal efficacy occurred between 7 and 30 days and lasted at least 3 months, with some improvement still noted at 6 months, by the injection with 100–300 U of type A toxin into detrusor muscle in the patients with urinary incontinence. It seems that all these trials were performed with commercial toxin-preparations, which are actually PTX.

In our present study, NTX alone was used instead of PTX for the treatment of the patients with both neurogenic and nonneurogenic detrusor overactivity. As for the advantage of NTX over PTX as a therapeutic agent, following two points might be proposed: (1) NTX works more quickly than PTX because the latter must dissociate into a NTX and a nontoxic component(s) for demonstrating its action, and (2) induction of neutralizing antibodies after repeating treatment with NTX is lower than that with PTX because some subcomponents of the latter, HA1 and HA3b, have adjuvant activity (Lee *et al.*, 2005). This time, excellent effects were demonstrated within 7 days after injection, and lasted from 3 to 12 months. The suitable treatment doses for idiopathic and for neurogenic patients were 100 and 200 U, respectively. Therefore, it seems that NTX may work slightly quicker than PTX. As a matter of course, to confirm this conclusion, we must perform a lot of more careful experiments. In an attempt to clarify the second postulation, a procedure for detecting small amount of antibodies in the sera is needed. We are now trying to establish a new technique for it and, after this is achieved, we will investigate these points by employing the NTX and PTX purified by us.

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Author contribution

J.-C.L. and T.Y. contributed equally to this work.

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