Production of anti-neurotoxin antibody is enhanced by two subcomponents, HA1 and HA3b, of *Clostridium botulinum* type B 16S toxin–haemagglutinin

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Clostridium botulinum type B strain produces two forms of progenitor toxin, 16S and 12S. The 12S toxin is formed by association of a neurotoxin (NTX) and a non-toxic non-haemagglutinin (NTNH), and the 16S toxin is formed by conjugation of the 12S toxin with a haemagglutinin (HA). HA consists of four subcomponents designated HA1, HA2, HA3a and HA3b. When mice were immunized with formalin-detoxified NTX, 12S or 16S, a significantly greater amount of anti-NTX antibody (Ab) was produced in the mice injected with 16S than in NTX- or 12S-injected mice. Immunization with NTX mixed with HA1 and/or HA3b also increased the anti-NTX Ab production, whereas NTX mixed with HA2 did not, indicating that HA1 and HA3b have adjuvant activity. This was further confirmed by immunizing mice with human albumin (Alb) alone or Alb mixed with either HA1 or HA3b. When mouse-spleen cells were stimulated with NTX, 16S or different HA subcomponents, 16S, HA1, HA3b and the mixture of HA1 and HA3 significantly increased interleukin 6 (IL6) production compared with NTX alone. Transcription of IL6 mRNA was low after stimulation with NTX alone, but increased to 16S-stimulation levels when NTX was mixed with HA1 or HA3b. In flow cytometry using labelled Abs against CD3 and CD19, the percentage of CD19 cells was higher following stimulation with 16S or NTX mixed with HA1 or HA3b compared with stimulation with NTX. The percentage of CD3 cells remained unchanged. These results suggest strongly that HA1 and HA3b demonstrate adjuvant activity via increasing IL6 production.

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INTRODUCTION

The neurotoxins (NTXs) produced by *Clostridium botulinum* are considered to be the most potent toxins known. Molecular mass of the NTXs is approximately 150 kDa and they are classified into seven groups, types A–G.

Abbreviations: Alb, human albumin; HA, haemagglutinin; NTNH, non-toxic non-haemagglutinin; NTNH–HA, non-toxic component; NTX, neurotoxin; PTX, progenitor toxin.

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Endogenous protease(s) from the bacteria cleave the single-chain NTXs into a dichain structure linked with a disulfide bond. Therefore, NTXs can be separated into two fragments, designated a light chain (L, 50 kDa) and a heavy chain (H, 100 kDa) upon SDS-PAGE under reducing conditions (Sugiyama, 1980). *C. botulinum* type B culture produces both single (unnicked) and dichain (nicked) forms of NTX (Arimitsu *et al.*, 2003). In culture fluids, NTX is associated with non-toxic components to form progenitor toxin (PTX). Type B culture produces two different-sized PTXs, 16S (or L toxin, 500 kDa) and 12S (or M toxin, 300 kDa)

Fig. 1. SDS-PAGE and Western blot analyses. (a, b) SDS-PAGE of the purified toxins, NTNH-HA and recombinant HA subcomponents. Molecular mass markers (in kilodaltons) are on the left (lane M) of the gel. (a) Type B NTX (lane 1), NTNH-HA (lane 2), 12S (lane 3), and 16S (lane 4). (b) Three recombinant GST-free HA subcomponents: HA1 (lane 1), HA2 (lane 2) and HA3b (lane 3), and 16S (lane 4). (c, d) Western blot of the gel shown in (b) (lanes 1–4). The blot was probed with anti-16S serum (c) or the mixture of anti-HA1, -HA2 and -HA3b sera (d). (e) Schematic of toxins based on gene organization (Fujinaga *et al.*, 1994; Oguma *et al.*, 1999).

(see toxin schematic in Fig. 1e). The 12S toxin consists of an NTX and a non-toxic component with no haemagglutinin (HA) activity and is designated non-toxic non-HA (NTNH). The 16S toxin contains HA in addition to 12S toxin. HA consists of four subcomponents with molecular masses of 51, 34, 19–23 and 18 kDa, designated HA3b, HA1, HA3a and HA2, respectively (Arimitsu *et al.*, 2003; Fujinaga *et al.*, 1994; Oguma *et al.*, 1999). The NTNH of the 16S toxin is intact (130 kDa), whereas that of the 12S toxin is cleaved at its N-terminal region to form 112 and 15 kDa bands upon SDS-PAGE with a reducing reagent (Arimitsu *et al.*, 2003; Oguma *et al.*, 1999). In alkaline conditions (above pH 7.2), PTXs dissociate into an NTX and a non-toxic component, the 12S toxin dissociates into an NTX and an NTNH, and the 16S toxin dissociates into an NTX and a non-toxic component that is a complex of NTNH and HA.

Recently, type A and B PTXs have been used for treating patients with many forms of dystonia (Jankovic & Brin,

1991; Schantz & Johnson, 1994; Scott, 1980). In both toxin types, PTXs are used because they are more stable than NTXs. The treatment is very effective, but has serious side effects for some patients in whom anti-PTX, including anti-NTX antibodies (Abs), is produced after several injections (Dressler *et al.*, 2003; Herrmann *et al.*, 2004). Although we have no quantitative data, there is some evidence that high levels of anti-NTX Ab are produced by immunization of rabbits with PTX alone compared with NTX alone. We believed this to indicate that PTX may have adjuvant activity. As HA1 and HA3b, but not HA2 or HA3a, bind specifically to the glycoprotein or glycolipid of erythrocytes and epithelial cells of the small intestine (Fujinaga *et al.*, 2000, 2004; Inoue *et al.*, 2001, 2003; Nishikawa *et al.*, 2004), we speculated that HA1 and HA3b might have adjuvant activity. In this study, we first observed that when mice were immunized with formalin-detoxified NTX, 12S or 16S, a significantly greater amount of anti-NTX Ab was produced in the mice injected with 16S than in NTX- or 12S-injected

mice. Thereafter, we demonstrated, by employing recombinant HA subcomponents, that immunization with NTX mixed with HA1 and/or HA3b also increased anti-NTX Ab production, whereas NTX mixed with HA2 did not. Adjuvant activity of HA1 and HA3b was also confirmed by employing human albumin (Alb) instead of NTX.

The mechanism of the adjuvant activity of HA1 and HA3b was investigated by *in vitro* testing. As Ab production is controlled by many cytokines, the level of cytokines produced by normal spleen cells that had undergone stimulation with HA1 or HA3b was assayed. Of the many cytokines, interleukin 4 (IL4), IL6 and gamma interferon (IFN- γ) were considered because of the limit of preparation volume and their significance in Ab production: IL4 is known as a B-cell growth factor (Paul, 1991), IL6 is a pleiotropic cytokine that plays a crucial role in the immune response, including differentiation of B cells into immunoglobulin-secreting cells (Hirano, 1998), and IFN- γ has a role in immunoregulation and macrophage activation, as well as in autoimmunity and cell-mediated cytotoxicity (Bach *et al.*, 1997). As IL6 production was increased significantly by HA1 and HA3b (as shown in Results), its mRNA transcription level and type of proliferated cells were then determined. Furthermore, as production of IL6 or other cytokines is mediated through several mitogen-activated protein kinase (MAPK) pathways [signal-regulated kinase (ERK) 1/2, p38 MAPK, phosphatidylinositol 3-kinase (PI3-K) and protein kinase A (PKA)], the effects of several inhibitors of these pathways on IL6 production were also observed (Arbibe *et al.*, 2000; Birkenkamp *et al.*, 2000; Bode *et al.*, 1998; Dahle *et al.*, 2004; Tuyt *et al.*, 1999).

METHODS

Purification of toxins and a non-toxic component. The toxins (16S, 12S and NTX) and a non-toxic component (NTNH-HA) were purified from a *C. botulinum* serotype B proteolytic strain Lamanna (or Okra) according to the procedure reported previously that used an aminophenyl β -lactose gel column (E-Y Laboratories Inc.) (Arimitsu *et al.*, 2003). The cells were cultured by the dialysis tube method. The toxins were precipitated with 60% saturated ammonium sulfate, treated with protamine and then partially purified by an SP-Toyopearl 650 M column (Tosoh) equilibrated with 50 mM sodium acetate buffer (pH 4.2). The fractions containing both 16S and 12S toxins were pooled, dialysed against 10 mM sodium phosphate buffer (pH 6.0) and then applied to a lactose gel column equilibrated with the same buffer. After washing, the bound 16S toxin was eluted by using the same buffer containing 0.2 M lactose. As the 12S toxin appeared in the pass-through fractions, it was further purified by column chromatography with Sephacryl S-300 (Amersham Biosciences) equilibrated with 50 mM sodium phosphate buffer (pH 6.0). NTX and NTNH-HA were dissociated from the purified 16S toxin obtained by dialysing it against 10 mM sodium phosphate buffer (pH 8.0) and each protein was purified by reapplying to a lactose gel column at pH 8.0; the NTX passed through the column, whereas the non-toxic component bound to the column (the latter was eluted by using the same buffer containing 0.2 M lactose).

These three forms of toxins were made non-toxic by dialysing them for 7 days at 37 °C against 100 mM sodium phosphate buffer (pH 8.0) containing 0.6% formaldehyde.

Preparation of recombinant HA subcomponents. We first cloned the genes for whole HA from type B Lamanna and determined the whole nucleotide sequence (GenBank accession no. AB232927). Thereafter, each HA subcomponent was expressed in *Escherichia coli* (BL21) as a glutathione S-transferase (GST)-fusion protein. First, recombinant plasmids for each subcomponent were constructed. Templates were generated by PCR with purified DNA from type B Lamanna. All oligonucleotides were synthesized by ESPEC Oligo Service Corporation. The following primer sequences were used: BHA1-F, 5'-CCGGAATCCGGCATGGAACACTATTC-AACAATCCAAAA-3'; BHA1-R, 5'-CCGCTCGAGCGGTTATGGG-TTACTCATAGTCCATATC-3'; BHA2-F, 5'-CCGGAATCCGGCA-TGTCAGTGAAGAAGCTTTCTAC-3'; BHA2-R, 5'-CCGCTCG-AGCGGTTATATTTTCAAGTTTGAACATTTGAT-3'; BHA3b-F, 5'-CGCGGATCCGCGGTTAGTAGCACACAACGAGTATTG-3'; BHA3b-R, 5'-CCGCTCGAGCGGTTAATTAGTAATATCTATA-TGCAATTTTAT-3'.

The PCR fragments were digested as follows: BHA1, *EcoRI*-*XhoI*; BHA2, *EcoRI*-*XhoI*; BHA3b, *BamHI*-*XhoI*; and cloned into pGEX-6P-3 (Amersham Biosciences) restricted with the same enzymes. The sequences of the constructs were verified by using an ABI PRISM 3100 genetic analyser (Applied Biosystems). The resulting plasmids were then introduced into *E. coli* and the GST-fusion proteins were expressed and affinity-purified with glutathione-sepharose 4B (Amersham Biosciences). After dialysis against a cleavage buffer, GST was removed by a PreScission protease (Amersham Biosciences) treatment. Finally, the purified HA subcomponents were obtained by reapplying to the glutathione-sepharose 4B column. From these preparations, contaminating lipopolysaccharides were removed by two successive applications to Detoxi-Gel columns (Pierce). All preparations after this step contained <50 endotoxin units (mg protein)⁻¹, as determined in a Limulus amoebocyte-lysate assay (BioWhittaker) (de Haan *et al.*, 2002; Gao & Tsan, 2003).

SDS-PAGE and immunoblot analyses. SDS-PAGE was performed on a 12.5% separating gel according to the method of Laemmli (1970) and molecular masses were determined by using Perfect Protein Markers (6.5–200 kDa; Bio-Rad). Protein bands were visualized by staining with Coomassie brilliant blue (CBB) R-250 (Bio-Rad). For immunoblot analysis, proteins were separated by SDS-PAGE and then electroblotted onto PVDF membranes (Trans-Blot Transfer Medium; Bio-Rad) with a semi-dry blotting apparatus (Nippon Eido). After treating with 10% skimmed milk in PBS (PBS-skimmed milk) at 4 °C overnight and then washing with PBS containing 0.05% Tween 20 (PBS-Tween), the membrane was reacted for 1 h at 37 °C with 1:1000-diluted rabbit polyclonal Abs. Two different Ab preparations were used. One is anti-16S (toxoid) and the other one is the mixture of three sera against HA1, HA2 and HA3b, which had been prepared by immunization with each GST-free recombinant subcomponent. The membrane was then washed, treated with 1:10 000-diluted horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Jackson ImmunoResearch) for 1 h at 37 °C, washed again and, finally, the reacted bands were detected on a scientific imaging film (Kodak) by an enhanced chemiluminescence-Western blotting detection reagent (Amersham Biosciences).

Immunization protocol. Detoxified NTX, 12S or 16S (2 μ g in 0.25 ml in 10 mM PBS, pH 7.4) and NTX mixed with an equal amount(s) of NTNH-HA or different HA subcomponents (total, 2 μ g in 0.25 ml) were employed as immunogens as listed in Fig. 2. Each preparation was injected subcutaneously six times at 1-week intervals into the dorsal side of ten female BALB/c mice (6–8 weeks

Fig. 2. Anti-NTX Ab production after immunization with different antigens. Detoxified toxins or the NTX mixed with NTNH-HA or different HA subcomponents were injected into mice at a concentration of 2 μ g in 0.25 ml, as described in Methods. After immunization, the serum of each mouse was diluted 1:100 and its total immunoglobulin (a) and IgG (b) levels against NTX were assayed by ELISA. Data shown are the means $\pm$ SD obtained from ten mice. * P < 0.01; ** P < 0.001.

of age; Charles River) that were maintained under specific-pathogen-free conditions in Okayama University vivarium. Also, Alb was used as immunogen alone or in combination with HA1, HA2 or HA3b. The amounts injected were the same as described above, but each preparation was injected into five mice three times at 1-week intervals (Fig. 3). Six days after the last injection, the mice were sacrificed and blood was collected for serum. This immunization schedule was based on the assumed treatment of patients with dystonia, i.e. recurrent injection of a low dose of antigen (toxin).

Serum-Ab levels. The serum-Ab level of each mouse against NTX or Alb was determined by ELISA and the mean level of ten (Fig. 2) or five (Fig. 3) mice was obtained for each preparation. The wells of 96-well flat-bottom microplates (Cellstar) were coated with 1 μ g NTX or Alb in 0.1 ml 10 mM PBS (pH 7.4) at 4 $^{\circ}$ C overnight. After washing three times with PBS-Tween and blocking with PBS-skimmed milk for 2 h at 37 $^{\circ}$ C, mouse serum samples (0.1 ml) diluted 1:100 in PBS-skimmed milk were reacted for 1 h at 37 $^{\circ}$ C. After washing, they were then reacted with 0.1 ml secondary Abs for 1 h at 37 $^{\circ}$ C. As for the secondary Abs, 1:2000- or 1:5000-diluted

Fig. 3. Anti-Alb Ab production after immunization with different antigens. Alb or Alb mixed with HA subcomponents was used as immunogen at a concentration of 2 μ g in 0.25 ml. After immunization, the reaction of each mouse serum to Alb was assayed by ELISA as described above. Data shown are the means $\pm$ SD obtained from five mice. * P < 0.05; ** P < 0.01.

HRP-conjugated rabbit anti-mouse immunoglobulins (Dako) or anti-mouse IgG (ZYMED) were used. Colour was developed with 0.04% *o*-phenylenediamine (Wako). The reaction was stopped at 30 min by the addition of 1 M H_2SO_4 and A_{490} was read by using a microplate reader (Bio-Rad). In the cases immunized with NTX, 16S, NTX + HA1, NTX + HA2 or NTX + HA3b, the sera of 10 mice were pooled, diluted serially in two- or tenfold steps and then their end-point reaction titres were obtained.

Cytokine levels in splenocyte cultures. Spleen cells were removed aseptically from normal mice and adjusted to 4×10^6 cells ml^{-1} in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum. Cell suspension (0.1 ml) was distributed into each well of 96-well tissue-culture plates (Cellstar), followed by the addition of 0.1 ml of the different antigens (NTX, 16S, NTNH-HA, HA1, HA2, HA3b, HA1 + HA3b or HA1 + HA2 + HA3b) as shown in Figs 4 and 5, previously diluted with the medium to 2, 10, 20, 50 or 100 μ g ml^{-1} . After incubation for 48 h at 37 $^{\circ}$ C in a 5% CO_2 incubator, the supernatant of each well was collected separately and amounts of IFN- γ , IL4 and IL6 were assayed by quantitative sandwich enzyme immunoassay (AN'ALYZA). Three wells were

Fig. 4. IFN- γ production in spleen cells after stimulation with different antigens. Mouse-spleen cells (4×10^5 cells at 0.2 ml per well in 96-well plates) were incubated in the presence of different amounts of antigen (a, b) or 5 μ g antigen (c, d) at 37 °C for 2 days, and the amount of IFN- γ was assayed by a sandwich ELISA kit. Data shown are the means $\pm$ SD obtained from triplicate samples. * $P < 0.01$.

prepared for each antigen and their readings were averaged. Finally, these were expressed as protein amounts with reference to a standard curve prepared from the results of recombinant mouse cytokines.

IL6 mRNA RT-PCR. Spleen-cell suspension (0.5 ml per well; 4×10^6 cells ml⁻¹) was added to 24-well tissue-culture plates (Cellstar), followed by 0.5 ml of the different antigens in medium, NTX, 16S, NTX+HA1 or NTX+HA3b, at final concentrations of 12.5 or 25 μ g ml⁻¹ per well as shown in Fig. 6. After incubation for

1, 3, 6 and 24 h at 37 °C in 5% CO₂, the cells were collected by low-speed centrifugation and total RNA was then isolated by using RNAzol B (Tel-Test). cDNA was first prepared in a 20 μ l volume containing 10 μ l RNase-free H₂O, 4 μ l 5 $\times$ RT buffer, 2 μ l dNTP mixture, 1 μ l RNase inhibitor, 1 μ l oligo(dT)₂₀, 1 μ l ReverTra Ace (Toyobo) and 1 μ l RNA product, at 42 °C for 20 min, 99 °C for 5 min, then 4 °C for 5 min. Thereafter, RT-PCR was performed with ReverTra Dash (Toyobo), employing the cDNA thus prepared and the primers for mouse IL6 (Toyobo) according to the package protocol. Cycling parameters were as follows: 30 cycles of 98 °C for

Fig. 5. IL6 production in spleen cells after stimulation with different antigens. Mouse-spleen cells (4×10^5 cells in 0.2 ml per well in 96-well plates) were incubated in the presence of different amounts of antigen (a, b) or 5 μ g antigen (c, d) at 37 °C for 2 days, and the amount of IL6 was assayed by a sandwich ELISA kit. Data shown are the means $\pm$ SD obtained from triplicate samples. * $P < 0.05$; ** $P < 0.001$.

Fig. 6. IL6 mRNA RT-PCR. Mouse-spleen cells (2×10^6 cells ml^{-1} per well in 24-well tissue-culture plates) were cultured in the presence of different antigens (12.5 or 25 μg per well). After incubation for 1, 3, 6 or 24 h, RT-PCR for mouse IL6 was performed as described in Methods. As a control, mRNA for G3PDH was assayed. The PCR products of IL6 and G3PDH were 337 and 983 bp, respectively.

10 s, 60 °C for 2 s and 74 °C for 30 s. The primers for mouse IL6 were: 5'-CCAACTGGATATAATCAGGAAAT-3' (5' primer) and 5'-CTAGGTTTGCCGAGTAGATCTC-3' (3' primer), and the

expected product size was 337 bp. As a control, mRNA for mouse glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was assayed. The primers for G3PDH were: 5'-TGAAGGTCGGTGTGAACGGATTGGC-3' (5' primer) and 5'-CATGTAGCCATGAGGTCCACCAC-3' (3' primer), and the expected product size was 983 bp. PCR products were run on 2% agarose gels, stained with ethidium bromide and then viewed with a UV transilluminator.

Effects of signal-transduction inhibitors on IL6 production.

Four signal-transduction inhibitors of ERK1/2 (PD98059; 25 μM), p38 MAPK (SB202190; 25 μM), PI3-K (Ly294002; 25 μM) and PKA (KT5720; 1 μM) were purchased from Calbiochem and dissolved in DMSO solution to 25 or 1 μM as described above, and 2 μl of each preparation was added to spleen cells (100 μl) in 96-well culture plates (Dahle *et al.*, 2004). After incubation for 30 min, the cells were stimulated with 100 μl NTX, 16S, HA1 or HA3b (final concentration, 5 μg in 0.202 ml per well) and levels of IL6 produced were then assayed. As controls, cell cultures mixed with 2 μl DMSO solution and 100 μl RPMI 1640 medium with or without each stimulant were prepared (Fig. 7).

Flow cytometry. Cell suspension (1 ml; 4×10^6 cells ml^{-1}) was inoculated into six-well tissue-culture plates (Cellstar) and an equal volume of each antigen (25 or 50 μg) in medium was added. After incubation for 48 h at 37 °C, the cell culture in each well was transferred into a 15 ml centrifuge tube and reacted with 10 μl fluorescein isothiocyanate-conjugated anti-mouse CD3 or phycoerythrin-conjugated anti-mouse CD19 for 1 h at 4 °C. Thereafter, the cells were collected by low-speed centrifugation, washed with 10 mM PBS (pH 7.4), fixed in the same buffer containing 1% formaldehyde (Wako) and then analysed by a FACScalibur flow cytometer (Becton Dickinson); 10 000 live cells were collected from each sample and analysed by CellQuest software (Fig. 8).

Statistical analysis. Results were expressed as means $\pm$ SD. Data were compared by using Scheffe's multiple-comparison test and differences were considered significant at P values of <0.05 .

Fig. 7. Effects of signal-transduction inhibitors on IL6 production. Mouse-spleen cells were added with signal-transduction inhibitors 30 min prior to stimulation with antigens (final concentration, 5 μg in 0.202 ml). After incubation at 37 °C for 2 days, the amount of IL6 was assayed by a sandwich ELISA kit. As controls, cell cultures mixed with DMSO solution and RPMI 1640 medium with (C-2) or without (C-1) each stimulant were prepared. Data shown are the means $\pm$ SD obtained from triplicate samples. * $P < 0.05$; ** $P < 0.01$.

Fig. 8. Flow cytometry. Mouse-spleen cells (4×10^6 cells in 2 ml per well in six-well tissue-culture plates) were incubated in the presence of different antigens (25 or 50 μg) for 48 h at 37 °C. The whole cell culture in each well was transferred into a 15 ml centrifuge tube and reacted with labelled anti-mouse-CD3 or -CD19 Abs. Thereafter, the cells were analysed by a FACScalibur flow cytometer and 10 000 live cells from each sample were collected. Data were analysed by CellQuest software. (a) Means $\pm$ SD obtained from triplicate samples. * $P < 0.01$; ** $P < 0.001$. (b) Representative sample of the triplicate data.

RESULTS AND DISCUSSION

Preparation of toxins and recombinant HA subcomponents

The three forms of toxin (NTX, 12S and 16S) and a non-toxic component (NTNH-HA) were purified by column chromatography and the three subcomponents of HA (HA1, HA2 and HA3b) were prepared as recombinant proteins. The size and purity of these preparations were checked by SDS-PAGE (Fig. 1). The banding profiles of all preparations were those predicted based on their molecular composition and molecular mass, and some were reported previously (Arimitsu *et al.*, 2003). The NTX showed three main bands corresponding to whole (intact or unnicked) NTX (approx. 150 kDa), a heavy (H, 100 kDa) and a light (L, 50 kDa) chain of NTX (Fig. 1a, lane 1). The 12S toxin demonstrated five bands; three NTX bands and two bands of 106 and 15 kDa that are derived from NTNH (130 kDa) by cleavage at its N-terminal region (Fig. 1a, lane 3). The 16S toxin demonstrated four HA bands, HA1 (34 kDa), HA2 (18 kDa), HA3a (19–23 kDa) and HA3b (51 kDa), in addition to three NTX bands and one NTNH band (Fig. 1a and b, lane 4). As reported previously, the NTX light chain and the HA3b bands overlapped, and HA3a consisted of

several bands that had slightly different molecular masses (in this manuscript, HA3a was not used in all of the experiments because of this behaviour). NTNH showed only one band of 130 kDa, indicating that the NTNH of the 16S toxin is intact. The non-toxic component (NTNH-HA) demonstrated that all three NTX bands present in the 16S toxin had been eliminated (Fig. 1a, lane 2). Banding profiles of the recombinant HA subcomponents were as expected (Fig. 1b). An immunoblot analysis was performed by using two rabbit Ab preparations as described in Methods; one had anti-16S activity and the other was a mixture of three sera with activity against HA1, HA2 and HA3b. When the anti-16S serum was reacted with 16S toxin, all bands except for HA2 appeared clearly (Fig. 1c), as observed previously with type A (Inoue *et al.*, 1996). On the contrary, all bands including HA2 were demonstrated when the serum mixture was reacted with the HA subcomponents (Fig. 1d). As expected, the anti-16S serum reacted with recombinant HA subcomponents except HA2, but the serum mixture reacted with HA2, as well as with the other HA subcomponents. These results indicate that, although there is some reactivity of HA2 to the serum, its immunogenicity, as part of the native 16S toxin, is low, probably because HA2 is not exposed on the surface of the native toxin molecules.

Anti-NTX Ab production by immunization with different antigens

Each of the different antigen preparations [toxoids of NTX, 12S and 16S, NTX + non-toxic component (NTNH-HA), NTX + HA1, NTX + HA2, NTX + HA3b, NTX + HA1 + HA3b or NTX + HA1 + HA2 + HA3b] were injected into 10 mice and their immunoglobulin and IgG levels against NTX were determined by ELISA. The levels of both immunoglobulin and IgG differed only slightly among the 10 mice immunized with the same antigens and were averaged. As expected, the immunoglobulin level was higher than that of IgG and similar findings were obtained for the other antigen preparations. The mean Ab levels of the mice immunized with 16S or NTX + NTNH-HA were significantly higher than those of mice immunized with NTX alone or with 12S, even though the amounts of NTX in the preparations of 16S or NTX + NTNH-HA were low (in 16S toxin, the amount of NTX was estimated as approximately one-half to one-eighth that of NTX alone). Similarly, the Ab levels of mice immunized with NTX + HA1, NTX + HA3b, NTX + HA1 + HA3b or NTX + HA1 + HA2 + HA3b were higher than those immunized with NTX alone or NTX + HA2 (Fig. 2a and b). Following immunization with NTX, NTX + HA1, NTX + HA2, NTX + HA3b or 16S (2 µg), end-point ELISA titres were determined. For 16S, NTX + HA1 and NTX + HA3b, end-point titres were quite high (3200–6400) compared with NTX and NTX + HA2 (approx. 100). These data indicate that the immunogenicity of NTX is increased by HA, especially by HA1 and HA3b. Neither NTNH nor HA2 seemed to have such adjuvant activity. The Ab levels differed only slightly in the sera from mice immunized with NTX + HA1, NTX + HA3b or NTX + HA1 + HA3b, indicating that adjuvant activity of HA1 and HA3b is similar and the activity may not be increased dramatically by mixing HA1 and HA3b.

Anti-Alb Ab production by immunization with different antigens

Mice were immunized with Alb with or without mixing with one of the HA subcomponents (HA1, HA2 or HA3b) and their Ab levels against Alb were determined by ELISA. Each antigen was injected into five mice. The Ab levels of five mice immunized with the same antigens differed only slightly and were averaged. The mean Ab levels of the mice immunized with Alb + HA1 or Alb + HA3b were significantly higher than those of mice immunized with Alb alone or Alb + HA2 (Fig. 3a and b). Therefore, it was concluded that the immunogenicity of Alb is increased, again, by HA1 and HA3b.

Cytokine production from spleen cells stimulated with different antigens

Spleen cells obtained from non-immunized mice were incubated for 48 h in the presence of different antigens (NTX, NTNH-HA, 16S, HA1, HA2, HA3b, HA1 + HA3b or HA1 + HA2 + HA3b) in 96-well plates and the levels of IL6,

IL4 and IFN- γ were assayed. The level of IL4 differed only slightly for all of the antigen-stimulated preparations (data not shown), whereas IL6 levels were significantly different: HA1 > HA1 + HA2 + HA3b, HA1 + HA3b, 16S, HA3b > NTNH-HA, HA2 > NTX (Fig. 5). In the case of IFN- γ , production was clearly increased only by stimulation with 16S (Fig. 4). Therefore, it is strongly suggested that HA1, HA3b and 16S, which has these two HA subcomponents, demonstrate adjuvant activity mainly by increasing the production of IL6 from lymphocytes. The reason why only 16S increased IFN- γ production is not clear. This may be attributed to the tertiary structure of 16S having NTNH that is not formed by the preparations of NTX mixed with any HA subcomponents (the combination of NTX and HA does not occur without NTNH).

The transcription levels of mRNA for IL6 in the spleen cells stimulated with NTX, 16S, NTX + HA1 or NTX + HA3b were estimated by RT-PCR with G3PDH as a reference (Fig. 6). The levels of transcription were visibly increased in the presence of 16S (25 µg) and NTX (12.5 µg) mixed with equal amounts of HA1 or HA3b compared with NTX stimulation alone (12.5 or 25 µg). The specificity of IL6 production was further confirmed by observing the effects of signal-transduction inhibitors on IL6 production (Fig. 7). IL6 production with any stimulants was remarkably inhibited by SB202190, Ly294002 and KT5720, but not by PD98059. Our data show clearly that the IL6 production was mediated by p38 MAPK, PI3-K and PKA, but not by ERK1/2, in spleen cells.

Flow-cytometry analysis

As the production of IL6 in spleen cells was increased by stimulation with 16S, HA1 and HA3b, the type of proliferated cells was analysed by flow cytometry using labelled Abs against CD3 and CD19. The percentage of CD19-positive cells was higher following stimulation by 16S and NTX mixed with HA1 or HA3b compared with stimulation with NTX alone (Fig. 8). The percentage of CD3-positive cells remained unchanged (data not shown).

Based on these results, it is concluded that HA1 and HA3b have adjuvant activity, probably by increasing IL6 production. As both HA1 and HA3b are components of the 16S toxin molecule, this may explain why higher levels of anti-NTX Abs are induced following immunization with 16S than with NTX alone. IL6 production may be provoked either in monocytes that phagocytose HA1 and HA3b or in lymphocytes, or in both. Recently, we found that HA1 has two β -trefoil domains similar to the B-chain (lectin) of ricin, and that HA3b has the sialoadhesin (Siglec) family motif. The former binds to galactose and the latter binds to sialic acid of glycoprotein and/or glycolipid of red blood cells and epithelial cells of the small intestine (Fujinaga *et al.*, 2004; Inoue *et al.*, 2003). HA1 and HA3b may also bind to lymphocytes. Such binding might provoke the signal to produce IL6, leading to proliferation of Ab-producing cells (CD19-positive cells) and, finally, high levels of Ab

production. From the experiment using signal-transduction inhibitors, it became clear that p38 MAPK, PI3-K and PKA are involved in IL6 production. We are now investigating the detailed mechanism of how IL6 is produced by stimulation with HA1 and HA3b.

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REFERENCES

- Arbibe, L., Mira, J.-P., Teusch, N., Kline, L., Guha, M., Mackman, N., Godowski, P. J., Ulevitch, R. J. & Knaus, U. G. (2000). Toll-like receptor 2-mediated NF- κ B activation requires a Rac1-dependent pathway. *Nat Immunol* **1**, 533–540.
- Arimitsu, H., Inoue, K., Sakaguchi, Y., Lee, J., Fujinaga, Y., Watanabe, T., Ohyama, T., Hirst, R. & Oguma, K. (2003). Purification of fully activated *Clostridium botulinum* serotype B toxin for treatment of patients with dystonia. *Infect Immun* **71**, 1599–1603.
- Bach, E. A., Aguet, M. & Schreiber, R. D. (1997). The IFN γ receptor: a paradigm for cytokine receptor signaling. *Annu Rev Immunol* **15**, 563–591.
- Birkenkamp, K. U., Esselink, M. T., Kruijer, W. & Vellenga, E. (2000). An inhibitor of PI3-K differentially affects proliferation and IL-6 protein secretion in normal and leukemic myeloid cells depending on the stage of differentiation. *Exp Hematol* **28**, 1239–1249.
- Bode, J. G., Peters-Regehr, T., Schliess, F. & Häussinger, D. (1998). Activation of mitogen-activated protein kinases and IL-6 release in response to lipopolysaccharides in Kupffer cells is modulated by anisoosmolarity. *J Hepatol* **28**, 795–802.
- Dahle, M. K., Øverland, G., Myhre, A. E., Stuestøl, J. F., Hartung, T., Krohn, C. D., Mathiesen, Ø., Wang, J. E. & Aasen, A. O. (2004). The phosphatidylinositol 3-kinase/protein kinase B signaling pathway is activated by lipoteichoic acid and plays a role in Kupffer cell production of interleukin-6 (IL-6) and IL-10. *Infect Immun* **72**, 5704–5711.
- de Haan, L. D., Hearn, A. R., Rivett, A. J. & Hirst, T. R. (2002). Enhanced delivery of exogenous peptides into the class I antigen processing and presentation pathway. *Infect Immun* **70**, 3249–3258.
- Dressler, D., Bigalke, H. & Bencke, R. (2003). Botulinum toxin type B in antibody-induced botulinum toxin type A therapy failure. *J Neurol* **250**, 967–969 (corrected and republished as *J Neurol* **250**, 1263–1265).
- Fujinaga, Y., Inoue, K., Shimazaki, S. & 8 other authors (1994). Molecular construction of *Clostridium botulinum* type C progenitor toxin and its gene organization. *Biochem Biophys Res Commun* **205**, 1291–1298.
- Fujinaga, Y., Inoue, K., Nomura, T., Sasaki, J., Marvard, J. C., Popoff, M. R., Kozaki, S. & Oguma, K. (2000). Identification and characterization of functional subunits of *Clostridium botulinum* type A progenitor toxin involved in binding to intestinal microvilli and erythrocytes. *FEBS Lett* **467**, 179–183.
- Fujinaga, Y., Inoue, K., Watarai, S. & 10 other authors (2004). Molecular characterization of binding subcomponents of *Clostridium botulinum* type C progenitor toxin for intestinal epithelial cells and erythrocytes. *Microbiology* **150**, 1529–1538.
- Gao, B. & Tsan, M.-F. (2003). Recombinant human heat shock protein 60 does not induce the release of tumor necrosis factor α from murine macrophages. *J Biol Chem* **278**, 22523–22529.
- Herrmann, J., Geth, K., Mall, V. & 8 other authors (2004). Clinical impact of antibody formation to botulinum toxin A in children. *Ann Neurol* **55**, 732–735.
- Hirano, T. (1998). Interleukin 6 and its receptor: ten years later. *Int Rev Immunol* **16**, 249–284.
- Inoue, K., Fujinaga, Y., Watanabe, T., Ohyama, T., Takeshi, K., Moriishi, K., Nakajima, H., Inoue, K. & Oguma, K. (1996). Molecular composition of *Clostridium botulinum* type A progenitor toxins. *Infect Immun* **64**, 1589–1594.
- Inoue, K., Fujinaga, Y., Honke, K. & 7 other authors (2001). *Clostridium botulinum* type A haemagglutinin-positive progenitor toxin (HA⁺-PTX) binds to oligosaccharides containing Gal β 1-4GlcNAc through one subcomponent of haemagglutinin (HA1). *Microbiology* **147**, 811–819.
- Inoue, K., Sobhany, M., Transue, T. R., Oguma, K., Pedersen, L. C. & Negishi, M. (2003). Structural analysis by X-ray crystallography and calorimetry of a haemagglutinin component (HA1) of the progenitor toxin from *Clostridium botulinum*. *Microbiology* **149**, 3361–3370.
- Jankovic, J. & Brin, M. F. (1991). Therapeutic uses of botulinum toxin. *N Engl J Med* **324**, 1186–1194.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Nishikawa, A., Uotsu, N., Arimitsu, H. & 8 other authors (2004). The receptor and transporter for internalization of *Clostridium botulinum* type C progenitor toxin into HT-29 cells. *Biochem Biophys Res Commun* **319**, 327–333.
- Oguma, K., Inoue, K., Fujinaga, Y., Yokota, K., Watanabe, T., Ohyama, T., Takeshi, K. & Inoue, K. (1999). Structure and function of *Clostridium botulinum* progenitor toxin. *J Toxicol Toxin Rev* **18**, 17–34.
- Paul, W. E. (1991). Interleukin-4: a prototypic immunoregulatory lymphokine. *Blood* **77**, 1859–1870.
- Schantz, E. J. & Johnson, E. A. (1994). Preparation and characterization of botulinum toxin type A for human treatment. In *Therapy with Botulinum Toxin*, pp. 41–49. Edited by J. Jankovic & M. Hallett. New York: Marcel Dekker.
- Scott, A. B. (1980). Botulinum toxin injection into extraocular muscles as an alternative to strabismus surgery. *Ophthalmology* **87**, 1044–1049.
- Sugiyama, H. (1980). *Clostridium botulinum* neurotoxin. *Microbiol Rev* **44**, 419–448.
- Tuyt, L. M. L., Dokter, W. H. A., Birkenkamp, K., Koopmans, S. B., Lummen, C., Kruijer, W. & Vellenga, E. (1999). Extracellular-regulated kinase 1/2, Jun N-terminal kinase, and c-Jun are involved in NF- κ B-dependent IL-6 expression in human monocytes. *J Immunol* **162**, 4893–4902.