Vaccination with Recombinant Whole Heavy Chain Fragments of *Clostridium botulinum* Type C and D Neurotoxins

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Mice and ducks were subcutaneously immunized with recombinant whole heavy (H) chains of *Clostridium botulinum* type C and D neurotoxins, which were expressed as glutathione S-transferase fusion proteins. In the case of mice, it was confirmed that two immunizations with type C- and D-H chains, 10 μg each time, significantly increased the specific antibodies against 100-kDa H chains of type C and D neurotoxins in an immunoblot analysis and an enzyme-linked immunosorbent assay, respectively. The mice immunized with type C- and D-H chains showed no symptoms of botulism when they were challenged with C- and D-16 S toxins at doses, given intraperitoneally, of up to 104 and 103 minimum lethal doses (MLD), respectively, per mouse. Ducks were immunized with a total of 100 μg of type C-H chain. The ducks also developed specific antibodies to the type C-H chain and showed significant protection against a challenge with 104 duck MLD of C-16 S toxin given intravenously. These results indicate that recombinant whole H chains can be used as an effective and safe vaccine for type C and D botulism in domestic animals.

*Clostridium botulinum* strains produce immunologically distinct neurotoxins (types A to G) that inhibit the release of acetylcholine at the neuromuscular junctions and synapses. In type C and D strains, two different-sized progenitor toxins with molecular masses of approximately 500 kDa (16 S toxin) and 300 kDa (12 S toxin) are produced (15). Each toxin consists of neurotoxin and nontoxic components; 12 S toxin is a complex of neurotoxin and a nontoxic component showing no hemagglutinin (HA) activity, and 16 S toxin is a complex of 12 S toxin and HA. The neurotoxin consists of a light chain (50 kDa; L chain) and a heavy chain (100 kDa; H chain) joined by a single disulfide bond (2). L chain is a catalytic domain of the neurotoxin, whereas H chain has two domains, the amino-terminal half (HN) and the carboxy-terminal half (HC), which are associated with internalization (or translocation) and binding to the receptor on the neuron, respectively (13, 16).

Type C and D toxins provoke botulism in many animal species, including the avian form (14). In Japan, some farmers have used ducks, named “Aigamo” in Japanese, which are cross strain of Japanese Mallard and Khaki Campbell, for reducing the chemicals in the rice. Young ducks are released into a rice field to exterminate harmful insects or unwanted plants, grow up during the rice crop, and are finally used as meats after the harvest is finished. However, a few hundred ducks died of botulism in a certain area of Ishikawa prefecture. These ducks showed symptoms of leg and wing paralysis and became weak and listless. *C. botulinum* type C organisms were isolated from the contents of the gastric tract of the carcass and environmental materials such as soil, maggots, food, and (or) straw mats. We therefore planned to vaccinate these ducks.

At present, the most widely available vaccine for human and animals is formalin-inactivated toxoids. Although these are very effective, they are expensive and time-consuming to prepare and are slightly hazardous during detoxification. To solve these problems, a recombinant vaccine has been considered; HC of types A and F (1, 4) and a type C whole neurotoxin that becomes nontoxicigenic by modifying some amino acids in its active domain (8). Since it appears difficult to prepare a large amount of recombinant whole neurotoxin, we attempted to prepare recombinant HC. In a previous study, we prepared HC containing the histidine (His) tag of types C and D, and the vaccine effects were analyzed in mice (17). Protective effects were observed in both types C and D; however, their effects were not as significant as expected. Since it was thought that increased efficacy could be induced by injecting whole H chain (HN and HC; 100 kDa) rather than the HC, we have used here the whole recombinant type C- and D-H chains and studied their effectiveness in both mice and ducks.

**MATERIALS AND METHODS**

**Animals.** The mice (dfY strain, male, 6 to 8 weeks) were purchased from Shizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). They were kept in clean plastic cages laid with white flakes (Oriental Yeast Co., Ltd., Tokyo, Japan) and fed the MF certified diets (Oriental Yeast Co., Ltd.) and supplied water freely. The ducks (a cross of Japanese Mallard and Khaki Cambell, male and female, 3 weeks) were purchased from the Takahashi Hatching Farm (Osaka, Japan). The animals were kept in a yard and fed the Birdy balanced diet (Nippon Formula Feed Mfg. Co., Ltd., Yokohama, Japan) and water freely. All animal experiments was done in accordance with the animal experiment guidelines of Okayama University.
RESULTS

Preparation of recombinant GST fusion products and their SDS-PAGE profiles. The recombinant whole type C- and D-H immunization, and the specific antibody titers were checked by enzyme-linked immunosorbent assay (ELISA) and Western blotting tests as follows. ELISA. Flat-bottom 96-well plates were coated with 100 µl/well of 50-µg/ml (for duck serum test) or 10-µg/ml (for mice serum test) concentrations of 16 S toxin, followed by incubation overnight at 4°C. After the plate was washed three times with PBS containing 0.05% (vol/vol) Tween 20 (T-PBS), nonspecific binding was blocked by the addition of 200 µl of PBS containing 10% (vol/vol) skim milk (S-PBS) for 1 h at 37°C. After the plate was washed, 100 µl of sera from immunized animals that had been diluted serially with S-PBS was added, followed by incubation for 1 h at 37°C. Plates were again washed and further incubated for 1 h at 37°C with 100 µl of HRP-conjugated anti-serum antibodies (Dako, A/S, Copenhagen, Denmark), or 500-fold-diluted anti-duck IgG rabbit IgG/well as described above. After the plate was washed three times with T-PBS, the wells were reacted with 100 µl of citrate buffer (pH 5.0) containing 0.04% (wt/vol) of o-phenylenediamine and 0.02% (vol/vol) hydrogen peroxide for 30 min at 37°C. This reaction was stopped with 100 µl of 2 N H2SO4, and the absorbance measured at 490 nm in a NOVAPATH microplate reader (Bio-Rad, Hercules, Calif.).

Western blotting. The 16 S toxin was separated by SDS-PAGE according to the method of Laemmli (9) and then electroblotted onto polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, Mass.) with semidry blotting apparatus (Nippon Eido, Tokyo, Japan) as reported by Hirano and Watanabe (3). The membrane blocked the nonspecific binding of the protein with S-PBS for 2 h at 37°C. After it was washed with T-PBS, the membrane was reacted with 1,000-fold-diluted sera of immunized mice for 1 h at 37°C. After it was washed with T-PBS, the membrane was reacted with HRP-conjugated anti-mouse immunoglobulin rabbit IgG (Dako, A/S, Copenhagen, Denmark), or 500-fold-diluted anti-duck IgG rabbit IgG/well as described above. After the plate was washed three times with T-PBS, the wells were reacted with 100 µl of citrate buffer (pH 5.0) containing 0.04% (wt/vol) of o-phenylenediamine and 0.02% (vol/vol) hydrogen peroxide for 30 min at 37°C. This reaction was stopped with 100 µl of 2 N H2SO4, and the absorbance measured at 490 nm in a NOVAPATH microplate reader (Bio-Rad, Hercules, Calif.).

Preparation of recombinant IgG and HRP-conjugated rabbit IgG against duck IgG. Duck serum (10 ml) was saturated with 33% ammonium sulfate and left at 4°C overnight. After centrifugation at 15,000 × g for 20 min, the pellet was dissolved in 3 ml of phosphate-buffered saline (PBS; pH 7.4) containing 0.5 M NaCl and dialyzed against the same buffer. The sample was applied to a Sepharose S-300 (Amersham Biosciences) column (1.4 by 90 cm) equilibrated with the same buffer and 2-ml fractions of the second protein peak (immunoglobulin G [IgG] rich) were collected. After dialysis against 0.015 M sodium phosphate buffer (pH 6.3), the IgG-rich sample was applied to a DEAE-Toyopearl 650 M (Toyosh, Tokyo, Japan) column (1.0 by 5 cm) equilibrated with the same buffer. The proteins bound to the column were eluted with stepwise increases in NaCl concentration (0.05, 0.1, 0.15, 0.2, and 1 M), and IgG fractions confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were collected. The purified IgG was used to immunize a rabbit (New Zealand White, 13 weeks, female). The IgG (1 mg) was first mixed with Freund complete adjuvant and injected subcutaneously. Three weeks later, the IgG (1 mg) was mixed with Freund incomplete adjuvant and injected subcutaneously. After 2 weeks, serum was collected from the carotid artery, and IgG was purified by gel filtration as described for the purification of duck IgG, followed by protein A-Sepharose 4B (Amersham Biosciences) affinity column chromatography. The antibody thus obtained was then conjugated with horseradish peroxidase (HRP) by using an EZ-Link maleimide-activated HRP kit (Pierce, Rockford, Ill.).

Adjuvant. Aluminum hydroxide was used as the adjuvant of the vaccine. A portion, 6.6 g, of aluminum sulfate 14:18 water (Wako Pure Chemicals, Osaka, Japan) was dissolved in 10 ml of distilled water, and 60 ml of 1 N NaOH was added dropwise with gentle stirring with a magnetic stirrer at room temperature. After 10 min, the precipitate was centrifuged (1,000 × g, 10 min) and washed three times with distilled water. The precipitate was resuspended in 30 ml of PBS and mixed with a blender until the corpuscles remained suspended for more than 10 min. After concentration of the aluminum hydroxide, 100 µl of the slurry sample was dried and weighed. The concentration of the adjuvant was adjusted to 10 mg/ml with PBS, and thimerosal was added to a final concentration of 0.02%.

Immunization of animals. Mice and ducks were immunized according to the protocol shown in Table 1. As a negative control, PBS instead of the antigen was mixed with the adjuvant. Each antigen solution was injected subcutaneously into the back of the mouse (0.1 ml) or ducks (0.2 ml) and protected against oral infection, a second immunization was performed. Partial bleeding was performed after the tail vein (mouse) or basilic vein (duck) at 3 and 5 weeks after the primary immunization, and the specific antibody titers were checked by enzyme-linked immunosorbent assay (ELISA) and Western blotting tests as follows.
FIG. 1. SDS-PAGE profile of recombinant H chain fragments of type C and D neurotoxins. GST fusion proteins were extracted from transformed BL21 cells by sonication and partially purified by using a glutathione-Sepharose 4B column. GST was removed in some preparations, followed by analysis by SDS-PAGE. Lanes: 1, GST-fused C-H chain protein; 2, C-H chain protein with GST removed; 3, GST-fused D-H chain protein; 4, D-H chain protein with GST removed.

FIG. 2. (A) Antibody levels in antisera of mice immunized with recombinant C-H chain (C-H) and adjuvant alone (Cont.) against botulinum type C-16 S toxin. The sera were bled at 3 weeks after primary immunization (Once) and 2 weeks after secondary immunization (Twice). *, Significant difference ($P < 0.001$). (B) Specificity of the antisera of mice immunized with C-H chain against native botulinum C-16 S toxin in immunoblot analysis (lane 2). Lane 1 is the SDS-PAGE profile of botulinum type C-16 S toxin shown as a reference. H and L chains of neurotoxin are indicated with arrows, and the remaining bands indicate nontoxic components.
GST fusion products (HN plus HC, 100 kDa) were prepared in *Escherichia coli*. At first, the amounts of products were quite low probably because the length of inserted DNA might be too long for the host cells. However, a good expression system has finally been established. The produced recombinant type C- and D-H chains were purified by using an affinity column and then analyzed by SDS-PAGE. Both preparations demonstrated a main band with 126 kDa (Fig. 1, lanes 1 and 3) containing GST. After these preparations were cleaved GST with PreScission protease and successively purified with glutathione-Sepharose 4B, they showed bands with molecular masses of 100 kDa (Fig. 1, lanes 2 and 4). Some minor bands were also found in both proteins stained with Coomassie brilliant blue R-250. Since these bands did not react with rabbit anti-type C or D neurotoxins polyclonal antibodies, they were considered as products derived from host cells (BL21). From the viewpoint of cost, these contaminants and GST were not eliminated from the expression proteins for the following vaccine experiments.

**Antibody response of mice sera against botulinum toxins.** Anti-C- or D-H-chain antibody titers in the sera of mice bled at 3 weeks after the primary immunization and 2 weeks after the second immunization were measured by ELISA using C- or D-16 S toxin. In both toxin types, the antibody levels were significantly increased after immunization with GST-H chains compared to the control groups (Fig. 2A, and 3A). The levels were also significantly higher in the sera of the twice-immunized group than those of the once-immunized group. The specificities of the antibodies were confirmed by immunoblot analysis against type C- or D-16 S toxins; the antisera from mice immunized with type C- or D-H chain reacted with only the 100-kDa band of the type C- or D-16 S toxin (Fig. 2B or 3B, respectively).

**Protective effect against a challenge with 16 S toxins in immunized mice.** The mice were challenged with lethal doses of the 16 S toxins. All five mice immunized with type C-H chain survived a 10⁵ mouse i.p. MLD of C-16 S toxin with no symptoms. However, four of the six mice challenged with a 10⁶ mouse i.p. MLD died, and the two surviving mice showed severe botulism. On the other hand, all five mice immunized with type D-H chain were completely protected even though they were challenged with a 10⁶ mouse i.p. MLD of D-16 S toxin (Table 2). When the mice that survived the challenge with type C and D toxins were then cross-challenged with 10 mouse i.p. MLD of D and C toxins, respectively, no mice survived (data not shown).

**Protective effect against 16S toxins in immunized ducks.** Since the efficacy of the recombinant vaccines was confirmed in mice, type C recombinant H chain was then used in ducks. Thirty ducks were immunized as described in Materials and Methods.

As shown in Fig. 4, the antibody level in ducks significantly increased after immunization similar to the result seen in mice. These ducks were then challenged with type C-16 S toxin. Prior
to the challenge, we tried to determine the MLD of type C-16 S toxin in ducks by both the oral and the i.v. routes. The MLD values for the duck oral and i.v. routes were $10^5$ and $10^3$ mouse i.p. MLD, respectively. Since a lot of toxin is needed in an oral challenge, we used i.v. injection as a challenge route. All seven immunized ducks resisted the challenge with $10^2$ duck i.v. MLD, but the survival rate decreased to 5 of 7 (71.4%) and 4 of 7 (57.1%) when the birds were challenged with $10^3$ and $10^4$ duck i.v. MLD. All of the control ducks receiving adjuvant alone died, even with a 10 duck i.v. MLD (Table 3).

### DISCUSSION

Effective recombinant type C and D vaccines have been prepared in the present study. Previously, we had prepared recombinant HC (50 kDa) of type C and D neurotoxins containing His tag by using *E. coli*. However, the vaccine effects were not as significant as expected. In the present study, we attempted to prepare recombinant whole type C- and D-H GST fusion products (HN plus HC, 100 kDa) in *E. coli*, and these recombinant whole H products were used as vaccines without removing GST.

In the case of mice, all animals immunized with the recombinant type C- and D-H chains produced antibodies reacting with only H chains of type C- and D-16 S toxins, respectively. Since a lot of toxin is needed in an oral challenge, we used i.v. injection as a challenge route. All seven immunized ducks resisted the challenge with 10 duck i.v. MLD, but the survival rate decreased to 5 of 7 (71.4%) and 4 of 7 (57.1%) when the birds were challenged with $10^2$ and $10^3$ duck i.v. MLD. All of the control ducks receiving adjuvant alone died, even with a 10 duck i.v. MLD (Table 3).

### TABLE 2. Result of challenge to mice immunized with recombinant type C- or D-H chain

| Vaccination group | Challenge dose (i.p. MLD) | No. of animals that survived (%) |
|-------------------|---------------------------|---------------------------------|
| Recombinant type C-H | $10^3$ | 5 / 5 (100) |
|                   | $10^4$ | 5 / 5 (100) |
|                   | $10^5$ | 5 / 5 (100) |
|                   | $10^6$ | 2 / 2 (33.3) |
| Adjuvant control  | $10^3$ | 3 / 0 (0) |
|                   | $10^4$ | 3 / 0 (0) |
|                   | $10^5$ | 2 / 0 (0) |

### TABLE 3. Result of challenge to ducks immunized with recombinant C-H chain

| Vaccination group | Challenge dose (i.p. MLD) | No. of animals that survived (%) |
|-------------------|---------------------------|---------------------------------|
| Recombinant type C-H | $10^3$ | 7 / 7 (100) |
|                   | $10^4$ | 7 / 5 (71.4) |
|                   | $10^5$ | 7 / 4 (57.1) |
| Adjuvant control  | $10^3$ | 5 / 0 (0) |
|                   | $10^4$ | 2 / 0 (0) |
|                   | $10^5$ | 2 / 0 (0) |

*All animals were injected with botulinum 16 S toxin i.v.*
homology of neurotoxins produced by these two strains is low, but some conserved amino acid sequences exist on HN regions as indicated in Fig. 5. Therefore, it was speculated that cross-neutralization may be caused by antibodies reacting with the epitopes existing on HN. This time, little cross-reaction was observed either in vitro (Western blotting analysis; data not shown) or in vivo (toxin challenge test), indicating that few antibodies reacting with HN are produced. We suggested the following two reasons for this phenomenon: the immunization level may be low compared to the study previously performed with rabbits and/or the GST molecule (26 kDa) may cover some epitopes of HN that exist close to the GST molecule, but not those of HC, which are far from the GST, inhibiting the contact of the epitopes with the immune cells.

Since the efficacy of the recombinant vaccines was confirmed in mice, type C recombinant H chain was then used in ducks. The serum antibody titers of ducks were significantly increased by two immunizations, as in mice. In the i.v. challenge test with type C-16 S toxin, all of the ducks survived the challenge with 10 duck i.v. MLD (estimated 3.2/11003 g, corresponding to ca. 3/11003 mouse i.p MLD), and more than half of the ducks survived against 102 and 103 duck i.v. MLD. It appears that the exposure of ducks to such high titer toxins is rare in natural cases, indicating that recombinant whole type C-H chain can be used as an effective vaccine in ducks, too. If necessary, greater vaccination volumes and/or times should be used to increase the antibody titers.

In the present study, it became clear that whole H chains of

FIG. 5. Comparative alignments of the H-chain region of type C (C-St; upper) and D (D-1873; lower) neurotoxins. These sequences were derived from references 7 and 10. Asterisks and dots indicate the homologous and identical amino acid sequences, respectively.
type C and D can be used as safe and effective vaccines. However, in the case of avian, hundreds of animals need to be immunized by using a simple method. To resolve this problem, we are now studying different vaccination methods with different adjuvants and routes.

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