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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 10^5 and 10^6 minmum 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 10^3 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 nontoxigenic 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 (ddY strain, male, 6 to 8 weeks) were purchased from Shimizu 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.

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Purification of toxins. The *C. botulinum* type C strain, C-Stockholm (C-St), and the type D strain, D-1873, were used for the production and purification of type C and D toxins, respectively, according to the procedure of Inoue et al. (5, 6).

Construction of expression plasmid. The following primers were designed to amplify the nucleotides encoding amino acids Asp^{442} through Glu^{1291} and Asp^{446} through Glu^{1276} of the type C- and D-H chain fragments, respectively (7, 10): C-7SH Bam-1f (5′-CGC GGA TCC GCG GAT GGT AGA TCA TTA TAT AAT AAA ACA T-3′), C-7SH Xho-1r (5′-CCG CTC GAG CGG TTA TTC ACT TAC AGG TAC AAA ACC C-3′), D-7SH EcoR-1f (5′-CCG GAA TTC CGG CGA TGA TTC AAC ATG TAT TAA AGT TAA AA-3′), and D-7SH Xho-1r (5′-CCG CTC GAG CGG TTA CTC TAC CCA TCC TGG ATC CC-3′). The underlined restriction enzyme sites were incorporated into the primers (type C [BamHI and XhoI] and type D [EcoRI and XhoI]). Purified DNAs from C-St and D-1873 were used as templates for amplification by PCR. The PCR product encoding type C-H chain was restricted with BamHI and XhoI, and that of type D-H chain was restricted with EcoRI and XhoI. The products were purified from agarose gel electrophoresis bands by using a QIAquick gel extraction kit (Qiagen, Chatsworth, Calif.) and then inserted into an expression vector pGEX-6P-3 (Amersham Biosciences, Piscataway, N.J.) restricted with the same enzymes.

Protein expression and purification. The plasmids constructed as described above were transformed into competent cells (BL21). The cells were cultured in 400 ml of Luria-Bertani broth containing 50 μg of ampicillin/ml (final concentration) at 37°C until the optical density at 600 nm reached 0.5. After induction with a 0.1 mM final concentration of IPTG (isopropyl-β-D-thiogalactopyranoside), the cells were cultured for 30 h at 25°C. The cells were collected by centrifugation at 6,500 × *g* for 20 min, lysed by sonication, and centrifuged again at 15,000 × *g* for 20 min. From these lysates, the glutathione *S*-transferase (GST) fusion proteins were affinity purified by glutathione-Sepharose 4B as recommended by the supplier (Amersham Biosciences). GST tag was eliminated by treating the fusion proteins with PreScission protease (Amersham Biosciences) and reapplying them to the glutathione-Sepharose 4B column.

Preparation of duck 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 Sephacryl 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 650M (Tosoh, 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. To calculate the concentration of the aluminum hydroxide slurry, 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 dorsal side of the mice (0.1 ml) or ducks (0.2 ml). At 3 weeks postimmunization, a second immunization was performed. Partial bleeding was performed via the tail vein (mice) 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.

TABLE 1. Immunization plan for GST–H-chain protein in mice and ducks

Group	Antigen concn (μg/ml)	Adjuvant concn (mg/ml)	Injected		No. of animals vaccinated
			Vol (ml)	Protein amt (μg)	
For mice vaccination					
Recombinant type C-H	100	2	0.1	10	23
Recombinant type D-H	100	2	0.1	10	26
Adjuvant control		2	0.1	0	25[a]
For duck vaccination					
Recombinant type C-H	250	5	0.2	50	21
Adjuvant control		5	0.2	0	9

[a] Total number of animals used in type C- and D-H-chain experiments.

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% (wt/vol) skim milk (S-PBS)/well for 2 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 second antibodies, 1,000-fold-diluted 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 H_2SO_4, 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 the 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) for 1 h at 37°C. After the membrane was washed, the reacted band was detected on an X-ray film by an enhanced chemiluminescence-Western blotting detection reagent (Amersham Biosciences).

Determination of toxin MLD. The 16 S toxin was diluted in serial 10-fold steps with 20 mM sodium phosphate buffer (pH 6.0), and 0.5 ml of each dilution was injected intraperitoneally (i.p.) and intravenously (i.v.) into at least two mice and ducks, respectively. The animals were observed for 1 week, and deaths were recorded. The minimum lethal doses (MLD) per milliliter (mouse i.p. MLD/ml or duck i.v. MLD/ml) were calculated as the dilutions causing death in both animals.

Challenge of the toxin in animals. The mice were challenged i.p. with 10^3, 10^4, 10^5, and 10^6 MLD/0.5 ml of type C- or D-16 S toxin and observed for 1 week, and deaths were recorded. The mice that survived type C and D toxins were cross-challenged with type D- and C-16 S toxins, respectively.

All of the ducks were challenged i.v. with 10, 10^2, or 10^3 MLD/0.5 ml of type C-16 S toxin diluted with saline.

Statistical analysis. The antibody level in ELISA was statistically analyzed by using the Student *t* test between H chain and adjuvant groups or with the paired *t* test between once- and twice-immunized groups.

RESULTS

Preparation of recombinant GST fusion products and their SDS-PAGE profiles. The recombinant whole type C- and D-H

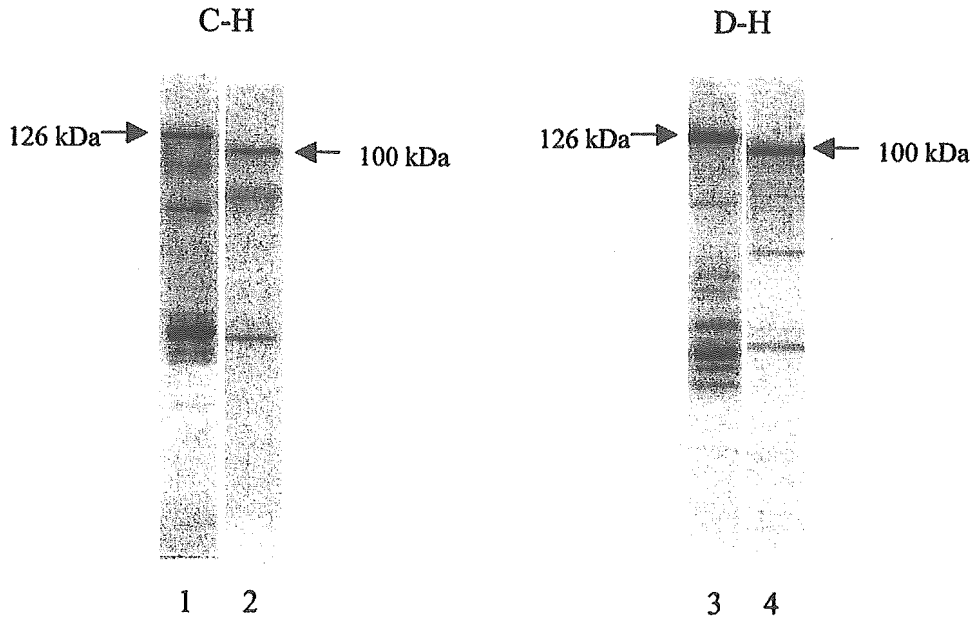

FIG. 1. SDS-PAGE profile of recombinant H chain fragments of type C and D neurotoxins. GST fusion proteins were extracted from transformed BL21 cells by sonication and partially purified by using a glutathione-Sepharose 4B column. GST was removed in some preparations, followed by analysis by SDS-PAGE. 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.

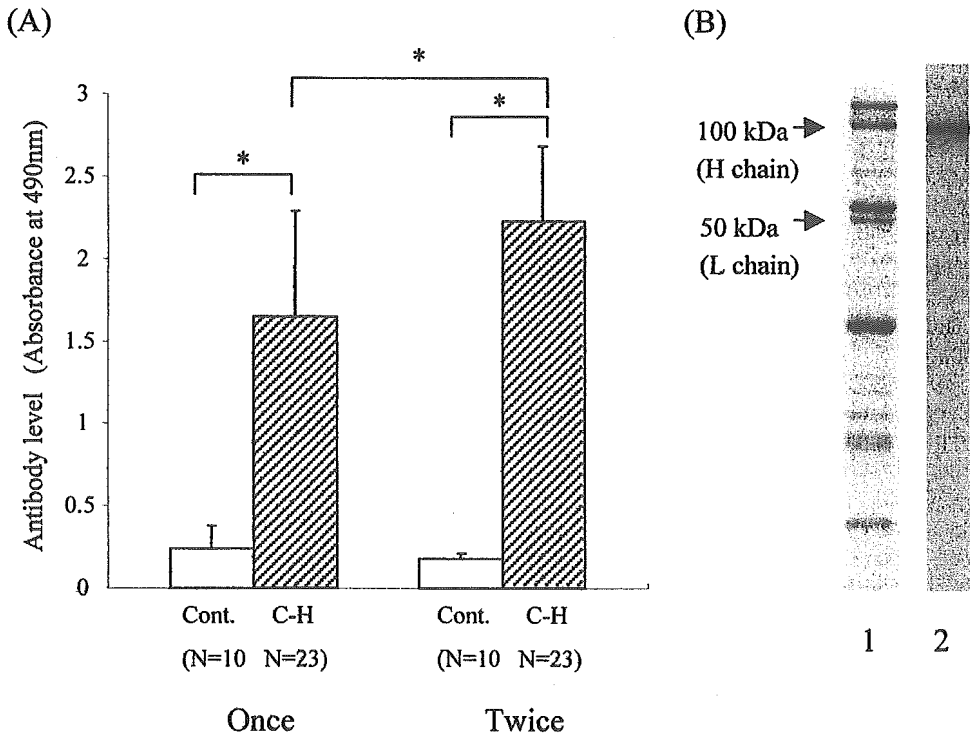

FIG. 2. (A) Antibody levels in antisera of mice immunized with recombinant C-H chain (C-H) and adjuvant alone (Cont.) against botulinum type C-16 S toxin. The sera were bled at 3 weeks after primary immunization (Once) and 2 weeks after secondary immunization (Twice). *, Significant difference ($P < 0.001$). (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.

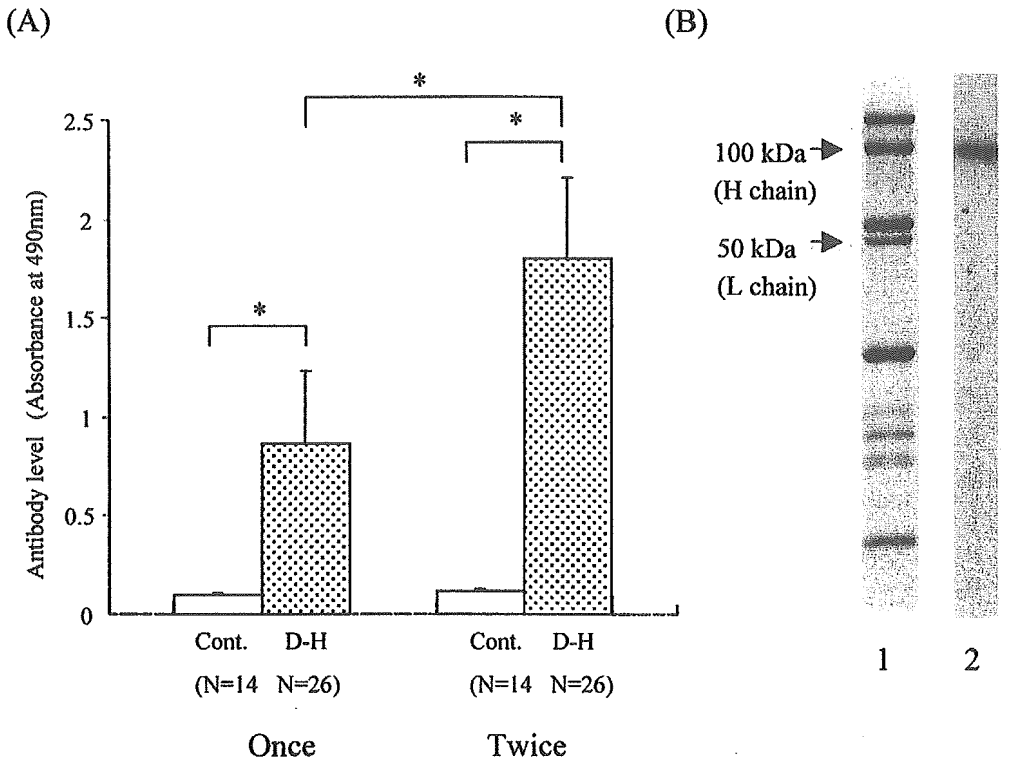

FIG. 3. (A) Antibody levels in antisera of mice immunized with recombinant D-H chain (D-H) and adjuvant alone (Cont.) against botulinum type D-16 S toxin. The mice were bled at 3 weeks after primary immunization (Once) and at 2 weeks after secondary immunization (Twice). *, Significant difference ($P < 0.001$). (B) Specificity of the antisera of mice immunized with D-H chain against native botulinum D-16 S toxin in immunoblot analysis (lane 2). Lane 1 is an SDS-PAGE profile of botulinum type D-16 S toxin shown as a reference. H and L chains 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, lanes 2), 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 10^5 mouse i.p. MLD of C-16 S toxin with no symptoms. However, four of the six mice challenged with a 10^6 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 10^6 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

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

Vaccination group	Challenge dose (i.p. MLD)	No. of animals challenged	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	6	2 (33.3)
Adjuvant control	10^1	3	0 (0)
	10^2	3	0 (0)
	10^3	2	0 (0)
Recombinant type D-H	10^3	5	5 (100)
	10^4	5	5 (100)
	10^5	5	5 (100)
	10^6	5	5 (100)
Adjuvant control	10^1	5	0 (0)
	10^2	5	0 (0)
	10^3	4	0 (0)

[a] All animals were injected with botulinum 16 S toxin i.p.

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 1 MLD values for the duck oral and i.v. routes were 1×10^5 and 3×10^3 mouse i.p. MLD, respectively. Since a lot of toxin is

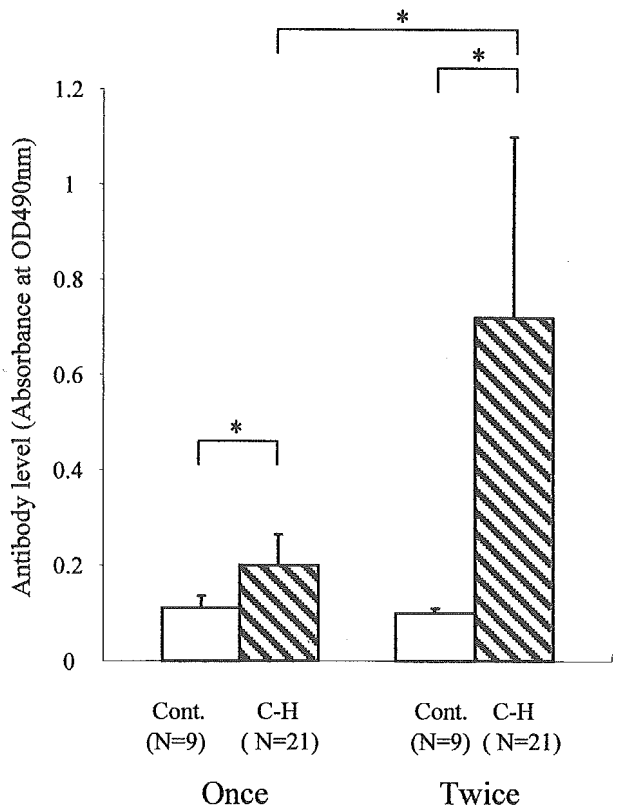

FIG. 4. Antibody levels in antisera of ducks immunized with recombinant type C-H chain (C-H) and adjuvant alone (Cont.) against botulinum type C-16 S toxin. The sera were bled at 3 weeks after primary immunization (Once) and 2 weeks after secondary immunization (Twice). *, significant difference ($P < 0.001$).

TABLE 3. Result of challenge to ducks immunized with recombinant C-H chain[a]

Vaccination group	Challenge dose (i.v. MLD)	No. of animals challenged	No. of animals that survived (%)
Recombinant type C-H	10^1	7	7 (100)
	10^2	7	5 (71.4)
	10^3	7	4 (57.1)
Adjuvant control	10^1	5	0 (0)
	10^2	2	0 (0)
	10^3	2	0 (0)

[a] All animals were injected with botulinum 16 S toxin i.v.

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).

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, in both ELISA and immunoblot analyses and resisted challenges with up to 10^5 and 10^6 mouse i.p. MLD of type C- and D-16 S toxins, respectively. Therefore, we concluded that both whole C- and D-H chains can be used as efficient vaccines. In previous experiments with C- and D-HC as immunogens, the production of antibodies was adequate, but their ability to neutralize 16 S toxins was somewhat low. Previously, the recombinant proteins were extracted from *E. coli* cells by sonication with a buffer containing 8 M urea, but this time they were extracted with only sonication. We therefore speculated that the reason for the low neutralization activity of the previous experiments was that the tertiary structure of the recombinant proteins denatured by urea treatment was not refolded effectively during dialysis, in addition to the shorter size of the product. We are now planning to prepare the recombinant HC extracted from *E. coli* cells only by sonication and check for its effectiveness as a vaccine.

We had also reported that the polyclonal antibodies prepared in rabbits by hyperimmunization with purified type C- and D whole H chains can cross-neutralize type D and C toxins to some extent, respectively, though the rates of cross-neutralization vary depending on the C and D strains (toxins) used (11, 12). This time, we used C-St and D-1873 strains. The

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C:442' DGRSLYNKTL DCRELLVKNT DLPFIGDISD VKTDIFLRKD INEETEVIYY PDNVSVDQVI
                     ***.  **...* .. .. .** .*  *..**.*  * .*. *.*. *
D:446"           D DSTCIKVKNN RLPYVADKDS ISQEIFENKI ITDETNVQNY SDKFSLDESI

C:502' LSKNTSEHGQ -LDLLYPSID SESEILPGEN QVFYDNRTQN VDYLNSYYYL ESQKLSDNVE
       *. ... . .  .* * *...  *.  ****.  ****. *.  ********** ******.***
D:497" LDGQVPINPE IVDPLLPNVN MEPLNLPGEE IVFYDDITKY VDYLNSYYYL ESQKLSNNVE

C:561' DFTFTRSIEE ALDNSAKVYT YFPTLANKVN AGVQGGLFLM WANDVVEDFT TNILRKDTLD
       ..*.* *.** **. *.*.** ..*.**.***  ***.****  ***.****** ***..*****
D:557" NITLTTSVEE ALGYSNKIYT FLPSLAEKVN KGVQAGLFLN WANEVVEDFT TNIMKKDTLD

C:621' KISDVSAIIP YIGPALNISN SVRRGNFTEA FAVTGVTILL EAFPEFTIPA LGAFVIYSKV
       ******.*** ********.* *. ****..* **..**..** *.******** **.*..**..
D:617" KISDVSVIIP YIGPALNIGN SALRGNFNQA FATAGVAFLL EGFPEFTIPA LGVFTFYSSI

C:681' QERNEIIKTI DNCLEQRIKR WKDSYEWMMG TWLSRIITQF NNISYQMYDS LNYQAGAIKA
       ***..***** .******.** *****.**.. .*****.*** *.*.****** *.***.****
D:677" QEREKIIKTI ENCLEQRVKR WKDSYQWMVS NWLSRITTQF NHINYQMYDS LSYQADAIKA

C:741' KIDLEYKKYS GSDKENIKSQ VENLKNSLDV KISEAMNNIN KFIRECSVTY LFKNMLPKVI
       ********** ********** ********** ********** ********** **********
D:737" KIDLEYKKYS GSDKENIKSQ VENLKNSLDV KISEAMNNIN KFIRECSVTY LFKNMLPKVI

C:801' DELNEFDRNT KAKLINLIDS HNIILVGEVD KLKAKVNNSF QNTIPFNIFS YTNNSLLKDI
       ****.** .* *..******* ********** .******.** .**.****** **********
D:797" DELNKFDLRT KTELINLIDS HNIILVGEVD RLKAKVNESF ENTMPFNIFS YTNNSLLKDI

C:861' INEYFNNIND SKILSLQNRK NTLVDTSGYN AEVSEEGDVQ LNPIFPFDFK LGSSGEDRGK
       ******.*** ********.* *.******** ***. ...** **.*.. *** *.***   .*
D:857" INEYFNSIND SKILSLQNKK NALVDTSGYN AEVRVGDNVQ LNTIYTNDFK LSSSG---DK

C:921' VIVTQNENIV YNSMYESFSI SFWIRINK-W VSNLPGYTII DSVKNNSGWS IGIISNFLVF
       .**. *.**. *...**. *. ****.*.*   ...  .**** .*...****. . * .. . .
D:914" IIVNLNNNIL YSAIYENSSV SFWIKISKDL TNSHNEYTII NSIEQNSGWK LCIRNGNIEW

C:980' TLKQNEDSEQ SINFSYDISN NAPGY-NKWF FVTVTNNMMG NMKIYINGKL IDTIKVKELT
       .*.. . . . *. *.*. *  . .** **** ***.***.**  **.****.*  .. *...*.
D:974" ILQDVNRKYK SLIFDYSESL SHTGYTNKWF FVTITNNIMG YMKLYINGEL KQSQKIEDLD

C:1039' GINFSKTITF EINKIPDTGL ITSDSDNINM WIRDFYIFAK ELDGKDINIL FNSLQYTNVV
        .....***.* *.  .. ..*  . ***** **.* **...****. ...    **.
D:1034" EVKLDKTIVF --------GI DENIDENQML WIRDFNIFSK ELSNEDINIV YEGQILRNVI

C:1099' KDYWGNDLRY NKEYYMVNID YLNRYMYANS RQIVFNTRRN NNDFNEGYKI IIKRIRGNTN
        ****** *.. ..***..* . *..**. ..* . .*.   .. .... .*  * .**.....
D:1086" KDYWGNPLKF DTEYYIINDN YIDRYIAPES NVLVLVQYPD RSKLYTGNPI TIKSVSDKNP

C:1159' DTRVRGGDIL YFDMTINNKA YNLFMKNETM YA--DNHSTE D-IYAIGLRE QTKDINDNII
         .*. .** .  ..*  *..  * ......*. **  ..... . .**. *.. . . . . *
D:1146" YSRILNGDNI ILHMLYNSRK YMIIRDTDTI YATQGGECSQ NCVYALKLQS NLGNYGIG-I

C:1216' FQIQPMNNTY YYASQIFKSN FNGENISGIC SIGTYRFRLG GDWYRHNYLV PTVKQGNYAS
        * *. . ..   * **** *. *.....     ..**..      ...*   ..*  .**..
D:1205" FSIKNIVSKN KYCSQIF-SS FRENTMLLAD IYKPWRFSF- ----KNAY-- TPVAVTNYET

C:1276' LLESTSTHWG FVPVSE
         * ***. *  *..
D:1257" KLLSTSSFWK FISRDPGWV
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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.

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 μg, corresponding to ca. 3×10^4 mouse i.p MLD), and more than half of the ducks survived against 10^2 and 10^3 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

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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Characterization of the Neurotoxin Produced by Isolates Associated with Avian Botulism

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SUMMARY. Several varieties of birds are affected by type C botulism. We conducted neutralization tests of culture supernatants of isolates from cases of avian botulism. Whereas the toxin produced by isolates derived from mammalian botulism was neutralized only with type C antitoxin, the toxins of all isolates related to avian botulism were neutralized with both type C and D antitoxins. An analysis of nucleotide sequences with several strains revealed that the neurotoxin gene in the isolates from avian botulism comprises two thirds of the type C neurotoxin gene and one third of the type D neurotoxin gene. This indicates that the neurotoxin of avian isolates is a mosaic of type C and D neurotoxins. We prepared three sets of primers to differentiate the gene for the mosaic form from the conserved genes of type C and D neurotoxins. The results of polymerase chain reaction with these primers indicated that all avian botulism–related isolates and specimens possess the gene for the mosaic form of the neurotoxin. The toxins purified from avian and mammalian isolates exhibited the same degree of lethality in mice, but the former showed greater toxicity to chickens than the latter. These results indicate that the mosaic neurotoxin is probably a pathogenic agent causing some forms of avian botulism.

RESUMEN. Caracterización de la neurotoxina producida por aislamientos asociados con botulismo aviar.

Varias especies de aves son afectadas por botulismo tipo C. Se realizaron pruebas de neutralización en sobrenadantes de cultivos provenientes de aislamientos de casos de botulismo aviar. Mientras que la toxina producida por aislamientos de botulismo humano fue neutralizada solamente con antitoxina tipo C, los aislamientos relacionados con botulismo aviar fueron neutralizados tanto con antitoxinas tipo C como tipo D. Un análisis de la secuencia de nucleótidos de varias cepas reveló que el gen de la neurotoxina en los aislamientos provenientes del botulismo aviar incluye dos tercios del gen de la neurotoxina tipo C y un tercio del gen de la neurotoxina tipo D. Esto indica que las neurotoxinas de los aislamientos aviares son una combinación de las neurotoxinas tipo C y D. Con la finalidad de diferenciar el gen para la forma combinada de los genes conservados de las neurotoxinas tipo C y tipo D, se desarrollaron tres pares de iniciadores. Los resultados de la prueba de reacción en cadena por la polimerasa indicaron que todos los aislamientos y especimenes relacionados con el botulismo aviar poseen el gen para la forma combinada de la neurotoxina. Las toxinas purificadas de aislamientos aviares y de mamíferos mostraron el mismo grado de letalidad en ratones, pero las toxinas aviares mostraron un mayor grado de toxicidad para los pollos que las de mamíferos. Estos resultados indican que la neurotoxina combinada es probablemente un agente patógeno causando algunas formas de botulismo aviar.

Key words: *Clostridium botulinum*, neurotoxin, avian botulism, neutralization, mosaic form, PCR

Abbreviations: BoNT = botulinum neurotoxin; FCMM = fortified cooked meat medium; H_C = carboxyl-terminal domain of heavy chain; H-chain = heavy chain; H_N = amino-terminal domain of heavy chain; IP = intraperitoneal; IV = intravenous; L-chain = light chain; L toxin = large-sized toxin; M toxin = medium-sized toxin; PCR = polymerase chain reaction; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SNARE = soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor

Clostridium botulinum is a gram-positive, spore-forming, and obligatory anaerobic bacterium, which is ubiquitous in substrate of rivers, lakes, marshes, and seas; in soils; and even in the intestines of animals (9). The organism has been classified into seven immunologic toxin types, A through G. Botulism is characterized by a generalized muscular weakness that extends gradually to all skeletal muscles and results in death from respiratory failure. Human botulism mainly involves types A, B, E, and F, and whereas the majority of cases of animal botulism are caused by type C, cattle and sheep are also particularly susceptible to botulism caused by type D (4).

In Japan, type C botulism occurred in Tokyo among wild ducks in 1972. Botulism among broiler chickens was first found in Miyazaki Prefecture on Kyushu Island in 1977 (1). Botulism in humans and domestic animals is usually caused by the ingestion of preformed toxins in food or feed. In avian botulism, type C toxin produced in birds or other animal carcasses is first ingested by fly larvae (2). If a healthy bird eats the toxic maggots, it develops symptoms of botulism after absorption of the botulinum toxin released from the maggots by the digestive tract. Spores of *C. botulinum* ingested together with the toxin proliferate in the carcass of the bird when it dies. Thus, more healthy birds are affected, and many birds may die in one outbreak (30). In an outbreak of botulism in broiler chickens, no source of preformed toxin was found, but a number of type C organisms were detected in feed, litter, and other specimens (25). It is therefore considered that toxin production in broiler chickens might take place *in vivo*, and the botulism in broilers could be categorized as a toxico-infection.

Botulinum neurotoxins (BoNT) are the causative agents of neuroparalytic disease. BoNTs share a common biochemical structure and mode of action. They are synthesized as single-chain peptides with a molecular mass of about 150 kD, which are proteolytically activated into a light chain (L-chain, 50 kD) and a heavy chain (H-chain, 100 kD) linked by a disulfide bond. It has been possible to assign some functional activities to certain domains of BoNT. The carboxyl-terminal domain of the H-chain (H_C) is implicated in the binding to the toxin receptor on the cell membrane, and the amino-terminal domain of the H-chain (H_N) is responsible for internalization of the

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Table 1. Origin of *Clostridium botulinum* type C and D strains and the results of toxin neutralization test and PCR.

Strain	Origin	Neutralization	PCR	GenBank accession no.
Stockholm	Mink	C	C-C	D90210
468	Horse	C	C-C	X72793
1873	Unknown	D	D-D	AB012112
CB-19	Mink	C	C-C	AB200358
003-9	Chicken	C/D[A]	C-D	AB200360
OFD-01	Chicken	C/D	C-D	AB200361
005-1	Pheasant	C/D	C-D	AB200362
OFD-02	Domestic duck	C/D	C-D	AB200363
OTZ01	Mandarin duck	C/D	C-D	AB200364
348	Chicken	C/D	C-D	
205-1	Pheasant	C/D	C-D	
005-6	Pheasant	C/D	C-D	
OFD-03	Domestic duck	C/D	C-D	
006-9	Domestic duck	C/D	C-D	
OTZ02	Ferruginous duck	C/D	C-D	
OTZ03	Little egret	C/D	C-D	
OTZ04	Tufted duck	C/D	C-D	
OTZ05	Little egret	C/D	C-D	
OTZ06	Little egret	C/D	C-D	
OTZ07	Little egret	C/D	C-D	

[A]The toxin in culture supernatant was neutralized with both C and D antitoxins.

light chain into the nerve cells. The light chain is an enzyme, identified as a zinc protease that acts on soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein, such as synaptobrevin, syntaxin, and SNAP-25. Cleavage of any of these proteins prior to SNARE bundle formation inhibits neurotransmitter release at the neuromuscular junction, leading to flaccid paralysis (26).

There are several reports on the determination of the complete primary structure of BoNT (19). Type C and D BoNT genes of several strains have been sequenced, and each BoNT gene possessed type-specific characteristics (3,10,11). However, the BoNT of type C strain 6813 has been reported to contain an H_C portion that is quite similar to the carboxyl-terminal one third of type D BoNT, indicating that the BoNT of strain 6813 is a mosaic form that consists of parts of type C and D BoNTs (20,21). Several attempts to determine the susceptibility of birds to type C and D toxin revealed that birds showed sensitivity to type C toxin rather than type D toxin (8). But there is no data available to evaluate the relationship between the mosaic BoNT and animal botulism. Originally, *C. botulinum* type C was divided immunologically into two subtypes, Cα and Cβ (12,22). The subtype Cα strains that produced C1, C2, and D toxic factors were usually associated with avian outbreaks, whereas the subtype Cβ strains produced only C2 toxic factor (13), which has been implicated in the disease affecting mammals, including cattle, minks, and horses (9). Since there is no report that type C strains produce two different types of BoNTs, these findings evoke the question of whether type C BoNT of the isolate implicated in avian botulism possesses properties different from those of BoNT produced by the isolate related to mammalian botulism. In this study, we found that BoNT derived from avian botulism exhibits the mosaic form, which is composed partly of the H_C of type D BoNT.

MATERIALS AND METHODS

Bacterial strains and specimens. The *C. botulinum* type C and D strains used in this experiment are listed in Table 1. Strains Stockholm 468 (23) and 1873 were used as references for type C and D, respectively. The other strains, with the exception of strain CB-19, were isolated from specimens of avian botulism, which had occurred in Japan. All strains were grown anaerobically with the fortified cooked meat medium (FCMM). The medium consisted of 12.5% cooked meat medium (Difco, Detroit, MI), 0.5% calcium carbonate, 1% ammonium sulfate, 1% yeast extract, 0.8% glucose, 0.5% soluble starch, and 0.1% cysteine-HCl and was adjusted to pH 7.6. After growing for 2 days at 37 C, the culture supernatant was subjected to toxin neutralization.

A mandarin duck (*Aix galericulata*), pochard (*Aythya ferina*), ferruginous duck (*Aythya nyroca*), tufted duck (*Aythya fuligula*), and little egret (*Egretta garzetta*), which died after presenting signs suggestive of botulism, were dissected to remove intestinal contents and liver, and their autopsy samples (0.5 g) were inoculated into 10 ml of FCMM. After heating at 70 C for 15 min, the media were incubated at 37 C for 2 days in an anaerobic jar. The culture supernatant was diluted appropriately with 0.05 M phosphate buffer, pH 6.2, containing 0.2% gelatin, 300 IU/ml penicillin and 0.5 mg/ml streptomycin, and was then injected intraperitoneally with an 0.5-ml dose into two mice. When the mice died, neutralization was carried out by mixing with an equal volume of antitoxin (Chiba Serum Institute, Chiba, Japan) of type C (1 IU/ml) or type D (10 IU/ml). Then two mice were injected with each antitoxin sample mixture. The mice were then observed for 4 days.

Nucleotide sequence of BoNT gene. *Clostridium botulinum* was inoculated into FCMM without calcium carbonate and was incubated overnight at 37 C in an anaerobic jar. After incubation, the culture supernatant (1.5 ml) was centrifuged at 12,000 × *g* for 1 min and the pellet was subjected to the extraction of total DNA with a PUREGENE DNA Isolation kit (Gentra Systems, Minneapolis, MN). The DNA fragments encoding the whole open reading frame of the BoNT gene were amplified by polymerase chain reaction (PCR) with the genomic DNA. The PCR primers (forward, 5′-ATGATATGTAATGACAATAACAAGG-3′; reverse, 5′-ACAGAGCAATCAGTATTTTACTACT-3′) were designed from the data of type C BoNT gene sequences (11,14). Following PCR amplification, the PCR products were purified using a GFX PCR DNA and a gel band purification kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The PCR products were directly sequenced using a BigDye terminator cycle sequencing kit (Perkin-Elmer, Boston, MA). The sequences were analyzed using the GENETYX computer program.

Differentiation of BoNT genes by PCR. The nucleotide sequences of primers (designated C1Fw, D1Fw, CDFw, C2Rv, D2Rv, and CDRv) were designed according to type C (strains Stockholm and 003-9) and type D (strain 1873) BoNT genes, as described below. The isolates and avian specimens were inoculated into FCMM without calcium carbonate and incubated overnight at 37 C under anaerobic conditions. The overnight culture (1.5 ml) was centrifuged at 12,000 × *g* for 1 min, and the pellet was suspended in 0.5 ml of 0.01 M sodium citrate (pH 7.0) containing 0.15 M NaCl. After washing three times by centrifugation, the pellet was finally suspended in 0.5 ml of sterile distilled water. After the suspension was heated at 99 C for 10 min, the supernatant was used as a template. PCR amplification was carried out in the reaction mixture (40 μl; template DNA [25–50 ng in 10 μl], 10 μM of each of the two primers [2 μl], 2.5 mM of each of the deoxynucleotide triphosphates [3.2 μl], Ex taq DNA polymerase [1 U; Takara, Tokyo, Japan] 25 mM $MgCl_2$ [1.6 μl], and ×10 Ex taq buffer [4 μl]). After heating for 2 min at 95 C, amplification at 95 C for 30 sec, 48 C for 30 sec, and 72 C for 1 min was performed for 30 cycles. PCR products were analyzed by electrophoresis through a 1.5% agarose gel and by staining with ethidium bromide.

Purification of toxins. *Clostridium botulinum* type C strains CB-19 and 003-9 and type D strain 1873 were used for the production and purification of type C and D toxins. The toxins were purified according to the method of Kurazano *et al.* (15). In the final step with gel filtration on Sephadex G-200, two different large (L)- and medium (M)-sized toxins were obtained, but M toxin was used in this study because M toxin possessed a specific toxicity that was at least twice as high as that of L toxin. The protein content was determined using the method of Lowry *et al.* (17). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 10% gel as described previously (16).

Determination of toxicity to mouse and chicken. Toxicity in mice was determined by intraperitoneal (IP) injection of 0.5-ml

Table 2. The results of neutralization tests and PCR on samples from birds with a preliminary diagnosis of botulism.

Species	Specimens	Neutralization	PCR
a. Mandarin duck	Cecum	C/D[A]	C-D
b. Pochard	Cecum	C/D	C-D
c. Ferruginous duck	Liver	C/D	C-D
d. Tufted duck	Cecum	C/D	C-D
e. Little egret	Small intestine	C/D	C-D

[A]The toxin in culture supernatant was neutralized with both C and D antitoxins.

volumes of serial dilutions into groups of four mice per dilution. The mice were observed for 4 days, and the 50% IP lethal dose per milliliter was calculated according to the method of Reed and Muench (24). The lethality in chickens was also determined by intravenous (IV) injection into wing veins of 2-wk-old White leghorn chicks (85–95 g weight, Hy-lineW77® strain).

RESULTS

Neutralization of avian botulism–derived toxin. In order to characterize the toxin produced by the isolates from avian botulism, we initially examined neutralization with type C and D antitoxins. The toxins of all 16 strains isolated from avian botulism were neutralized with both antitoxins, whereas strains CB-19 and 468, the isolates of mink or equine botulism, produced toxin that was only neutralized by type C antitoxin (Table 1). The culture supernatants of avian specimens were also neutralized with type C and D antitoxins. The observations indicate that avian botulism may be caused by organisms that produce toxin with both type C and D epitopes (Table 2).

Nucleotide sequences of BoNT genes in the isolates from avian botulism. We analyzed the BoNT genes of type C isolates that originated from different sources. The gene of strain CB-19 was amplified and the nucleotide sequence was determined (accession No. AB200358). The nucleotide and deduced amino acid sequences of CB-19/BoNT were identical to those of strains Stockholm and 468 from mammalian botulism (10). When the BoNT genes of five strains derived from avian botulism (003-9, OFD-01, 005-1, OFD-02, and OTZ01; accession Nos. AB200360, AB200361, AB200362, AB200363, and AB200364, respectively) were determined, we found that their nucleotide sequences were mostly identical to the sequence of strain 6813, which has the mosaic form of type C and D BoNTs (21). Their sequences had open reading frames of 3840 bp that encoded 1280 amino acids, and the predicted amino acid sequences are shown in Fig. 1. In a comparative analysis of their deduced amino acid sequences, the four (OFD-01, 005-1,

```
6813      1  MPITINNFNYSDPVDNKNILYLDTHLNTLANEPEKAFRIIGNIWVIPDRFSRDSNPNLNKPPRVTSPKSGYYDPNYLSTDSEKDTFLKEIIKLFKRINSR  100
OFD-01    1  ----------------------------------------------------------------------------------------------------  100
003-9     1  ----------------------------------------------------------------------------------------------------  100

6813    101  EIGEELIYRLATDIPFPGNNNTPINTFDFDVDFNSVDVKTRQGNNWVKTGSINPSVIITGPRENIIDPETSTFKLTNNTFAAQEGFGALSIISISPRFML  200
OFD-01  101  ----------------------------------------------------------------------------------------------------  200
003-9   101  ----------------------------------------------------------------------------------------------------  200

6813    201  TYSNATNNVGEGRFSKSEFCMDPILILMHELNHTMHNLYGIAIPNDQRISSVTSNIFYSQYKVKLEYAEIYAFGGPTIDLIPKSGRKYFEEKALDYYRSI  300
OFD-01  201  --------------------------------A---------------------------------------------------A---------------  300
003-9   201  --------------------------------A---------------------------N---------------------A---------------  300

6813    301  AKRLNSITTANPSSFNKYIGEYKQKLIRKYRFVVESSGEVAVDRNKFAELYKELTQIFTEFNYAKIYNVQNRKIYLSNVYTPVTANILDDNVYDIQNGFN  400
OFD-01  301  ----------------------------------------------------------------------------------------------------  400
003-9   301  ----------------------------------------------------------------------------------------------------  400

6813    401  IPKSNLNVLFMGQNLSRNPALRKVNPENMLYLFTKFCHKAIDGRSLYNKTLDCRELLVKNTDLPFIGDISDIKTDIFLSKDINVETEVIDYPDNVSVDQV  500
OFD-01  401  ----------------------------------------------------------------------------------------E-----------  500
003-9   401  ----------------------------------------------------------------------------------------E-----------  500

6813    501  ILSKNTSEHGQLDLLYPIIEGESQVLPGENQVFYDNRTQNVDYLNSYYYLESQKLSDNVEDFTFTTSIEEALDNSGKVYTYFPKLADKVNTGVQGGLFLM  600
OFD-01  501  ----------------------------------------------------------------------------------------------------  600
003-9   501  ----------------------------------------------------------------------------------------------------  600

6813    601  WANDVVEDFTTNILRKDTLDKISDVSAIIPYIGPALNISNSVRRGNFTEAFAVTGVTILLEAFQEFTIPALGAFVIYSKVQERNEIIKTIDNCLEQRIKR  700
OFD-01  601  ------------------------------------------G---------------------------------------------------------  700
003-9   601  ------------------------------------------G---------------------------------------------------------  700

6813    701  WKDSYEWMIGTWLSRITTQFNNISYQMYDSLNYQADAIKDKIDLEYKKYSGSDKENIKSQVENLKNSLDIKISEAMNNINKFIRECSVTYLFKNMLPKVI  800
OFD-01  701  ----------------------------------------------------------------------------------------------------  800
003-9   701  ----------------------------------------------------------------------------------------------------  800

6813    801  DELNKFDLKTKTELINLIDSHNIILVGEVDRLKAKVNESFENTIPFNIFSYTNNSLLKDIINEYFNSINDSKILSLQNKKNALVDTSGYNAEVRLEGDVQ  900
OFD-01  801  ----------------------------------------------------------------------------------------------------  900
003-9   801  ----------------------------------I-----------------------------------------------------------------  900

6813    901  VNTIYTNDFKLSSSGDKIIVNLNNNILYSAIYENSSVSFWIKISKDLTNSHNEYTIINSIKQNSGWKLCIRNGNIEWILQDINRKYKSLIFDYSESLSHT 1000
OFD-01  901  ----------------------------------------------------------------------------------------------------  1000
003-9   901  ----------------------------------------------------------------------------------------------------  1000

6813   1001  GYTNKWFFVTITNNIMGYMKLYINGRLKQSERIEDLNEVKLDKTIVFGIDENIDENQMLWIRDFNIFSKELSNEDINIVYEGQILRNVIKDYWGNPLKFD 1100
OFD-01 1001  ----------------------------------------------------------------------------------------------------  1100
003-9  1001  ----------------------------------D-----------------------------------------------------------------  1100

6813   1101  TEYYIINDNYIDRYIAPKSNILVLVQYPDRSKLYTGNPITIKSVSDKNPYSRILNGDNIMFHMLYNSGKYMIIRDTDTIYAIEGRECSKNCVYALKLQSN 1200
OFD-01 1101  ----------------------------------------------------------------------------------------------------  1200
003-9  1101  ----M--Y----------N--------S-I-----K-------AAN----------D-------D-RE----------TQ-GQ----------------  1200

6813   1201  LGNYGIGIFSIKNIVSQNKYCSQIFSSFMKNTMLLADIYKPWRFSFENAYTPVAVTNYETKLLSTSSFWKFISRDPGWVE                    1280
OFD-01 1201  ----------------------------------------------------------------------------------------------------  1280
003-9  1201  ----------------------------------------------------------------------------------------------------  1280
```

Fig. 1. Amino acid sequences of C/D mosaic BoNTs of strains 6813, OFD-01, and 003-9.

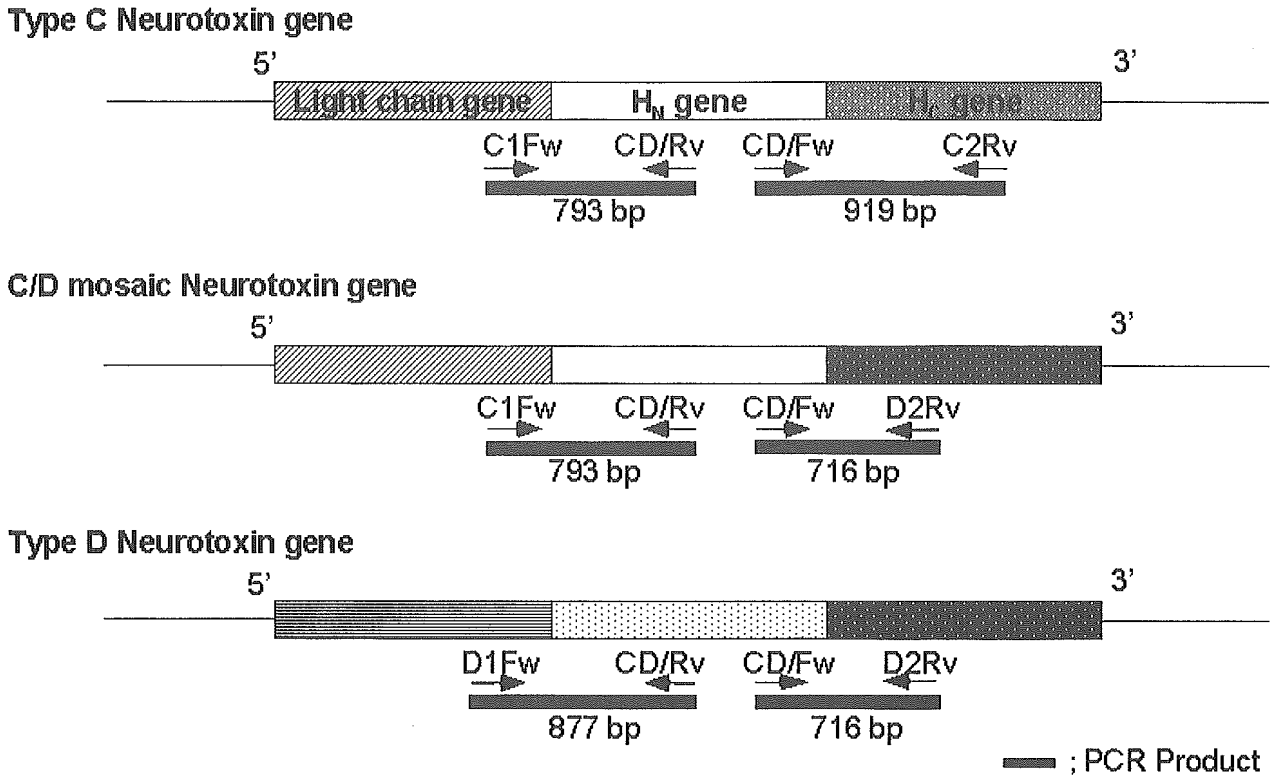

Fig. 2. Primers for PCR analysis and structures of type C and C/D mosaic and type D BoNT genes. Highly homologous domains have the same pattern.

OFD-02, and OTZ01) were completely identical to each other, while 003-9/BoNT showed 98.4% identity to the other four BoNTs. When compared with 6813/BoNT, the four BoNTs showed that the substitutions in the L-chain (residues 1–444) and H_N domain (residues 450–862) affected two residues (99.5% identity) in both domains. As for 003-9/BoNT, the amino acid substitutions in the L-chain and H_N domain concerned, respectively, three residues (99.3% identity), but those in the H_C domain affected 18 residues (95.7% identity). In 003-9/BoNT, the H_C domain was quite similar to that of type D 1873/BoNT (92.1% identity), whereas the L-chain and H_N domain shared only 46.9% and 72.3% identity to the respective domains of the type D. The amino terminal two thirds of 003-9/BoNT had 95.6% identity to type C Stockholm/BoNT (Fig. 2). We tentatively denoted the isolates encoding the C/D mosaic BoNT gene as C/D strain.

Characterization of BoNT derived from avian botulism by PCR. We constructed four pairs of primers to examine the characteristics of gene construction of BoNT by PCR (Fig. 2; Table 3). The primers were designed from type-specific nucleotide sequences in the L-chain (as the forward primer; C1Fw and D1Fw) and H_C domain (as the reverse primer; C2Rv and D2Rv) of type C and D BoNT. In the H_N domain, we selected two nucleotide sequences homologous to both BoNTs (CD/Fw and CD/Rv). In PCR, with the four pairs of primers for strain Stockholm (as the representative type C strain), PCR products of the expected size were obtained in lanes 1 and 3. For strain 003-9 (as the representative C/D strain) and strain 1873 (as the representative type D strain), the products were seen, respectively, in lanes 1 and 4 and lanes 2 and 4 (Fig. 3A). In further experiments with PCR, we found that all other isolates and specimens associated with avian botulism possessed the C/D mosaic BoNT gene, showing positive bands with pairs of primer C1Fw-CDRv and CD/Fw-D2Rv (Figs. 3B, 4).

Lethality of type C and C/D mosaic toxins to mouse and chicken. We next attempted to purify the toxins from the cultures of strains CB-19, 003-9, and 1873, respectively. SDS-PAGE analysis revealed that the purified M toxins of their strains were

Table 3. Location of primers within specified genes.

Primer[A]	Sequence[B]	Location within gene		
		Stockholm (C)	003-9 (C-D)	1873 (D)
C1Fw	5'-ATAAAGCAATAGATGGTAGAT-3'	1313–1333	1313–1333	—
D1Fw	5'-TTAATATAGAAAATTCGGGTCA-3'	—	—	1217–1238
CDFw	5'-GTTGGTGAAGTAGATAGATTAAA-3'	2476–2498	2476–2498	2464–2486
C2Rv[C]	5'-ATTTGTCGTGAGTTCGCATAC-3'	3372–3392	—	—
D2Rv[C]	5'-ATCTCTAATCCAAAGCATCTG-3'	—	3169–3189	3157–3117
CDRv[C]	5'-CCATCTYTTAAYYCTTTGTTC-3'	2083–2103	2083–2103	2071–2091

[A]The four pairs of primers (C1Fw and CD/Rv, CD/Fw and C2/Rv, CD/Fw and D2Rv, and D1Fw and CD/Rv) were used for PCR to differentiate BoNT genes. See Fig. 2.
[B]In the sequence of oligonucleotides, Y was substituted for C and T.
[C]Oligonucleotide positioned in the complementary strand.

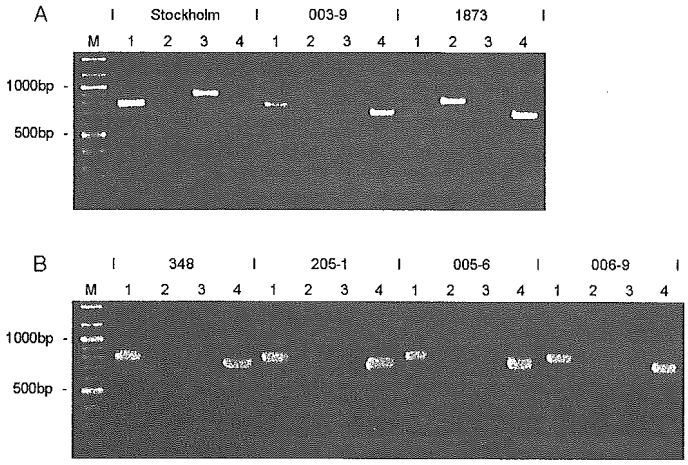

Fig. 3. Agarose gel electrophoresis of the PCR products of the strains with primer pairs. Lane 1: C1Fw and CDRv; Lane 2: D1Fw and CDRv; Lane 3: CDFw and C2Rv; and Lane 4: CDFw and D2Rv. M, molecular size standards (100-bp DNA ladder; BioLabs). (A) Strains Stockholm, 003-9, and 1879 were used as references for type C, C/D mosaic, and type D. (B) Four isolates (348, 205-1, 005-6, and 006-9) were subjected to PCR analysis as representatives of the isolates related to avian botulism listed in Table 1.

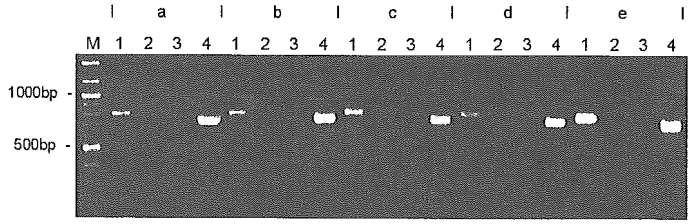

Fig. 4. Agarose gel electrophoresis of the PCR products of the incriminated samples of birds. The overnight cultures of specimens incriminated in avian botulism were used as the template for PCR. PCR was conducted with the primer pairs described in Lanes 1 to 4 in Fig. 3. Specimens listed in Table 2 (a–e) were used in this experiment.

separated into three bands (Fig. 5), the migration positions of which were corresponding to a nontoxic component, H-chain and L-chain (28). Since no attempt has been made to evaluate the extent to which the antigenic diversity between type C and C/D mosaic toxins affects their lethality in mice, we examined the toxicities of M toxins purified from strains CB-19 and 003-9 by a mouse IP injection method. The toxicities of CB-19 and 003-9 M toxins were 3.6×10^7 and 2.2×10^7 IP LD_{50}/mg protein, respectively. When the LD_{50} of chicken with M toxins was determined by an IV injection method, the lethality of 003-9 M toxin, equivalent to one IV LD_{50}, was 5.6×10^3 mouse IP LD_{50}, the value of which was about five times higher than that of CB-19 M toxin (2.6×10^4 mouse IP LD_{50}). In type D, the mouse lethality of purified M toxin (strain 1873) was 8.0×10^7 IP LD_{50}/mg protein, and one IV LD_{50} to chicken was estimated to be 1.4×10^6 mouse IP LD_{50}. These results indicated that type C toxins were more toxic to chicken than was type D toxin, a result that was in accord with the previous report (8).

DISCUSSION

Several varieties of birds, including wild ducks, waterfowls, and broiler chickens, are affected by type C botulism. Gross and Smith (8) reported that gallinaceous birds were highly susceptible to the culture supernatant of subtype Cα or Cβ strains but were resistant to that of type D strains. Dolman and Murakami (4) claimed that monkeys were susceptible to Cβ toxin rather than Cα toxin. The nomenclature for subtypes of the type C strains is obsolete, and the relationship between the immunologic cross-reactivity of the three toxic factors Cα, Cβ, and D and susceptibility in avian and mammalian remains to be elucidated (13).

There are several reports on the detection of type C BoNT genes by PCR (6,7,29). However, since a pair of primers designed by alignment of the DNA sequences of the L-chain were applied to the typing of isolates with the PCR products, the results did not provide any information on differentiating between typical type C and C/D mosaic BoNTs. In order to clarify the immunologic cross-reactivity among the subtypes in type C organisms, we first reexamined the results of neutralization tests of the culture supernatant of the isolates from cases of avian botulism and compared them with the results for mammalian isolates. Interestingly, the toxin produced by a mink isolate (strain CB-19) was neutralized with type C antitoxin but not type D antitoxin, while the toxins of all isolates related to avian botulism were neutralized with both type C and D antitoxins. The observations indicate that the toxin of the isolate implicated in avian botulism differs immunologically from the toxins of the isolates of mammalian botulism. In addition, we attempted to determine the whole nucleotide sequences of BoNT genes harbored in the isolates from avian botulism and found that they all comprise two thirds of the type C BoNT gene and one third of the type D BoNT gene, indicating that the BoNT of avian isolates is a mosaic form of type C and D BoNTs. Alternatively, the BoNT gene of the isolate of mink botulism accorded with a typical type C BoNT gene. These results are consistent with the observations that the isolates associated with avian botulism produced a single toxin that was neutralized with both type C and D antitoxins. In the following experiments, we examined whether avian isolates possess both type C and D genes or a mosaic gene consisting of parts of types C and D BoNT genes by use of PCR with four sets of primers. As expected, all avian botulism–related isolates and specimens exhibited PCR-positive bands, one of which was derived from the amino terminal portion of type C BoNT and another from the carboxyl-terminal portion of type D BoNT, indicating that their BoNTs constitute a mosaic of parts of type C and D BoNTs. These results indicate that the C/D mosaic toxin is probably produced by an avian botulism–related organism that had been considered to be subtype Cα (5,9). Recently, mass spectrometric

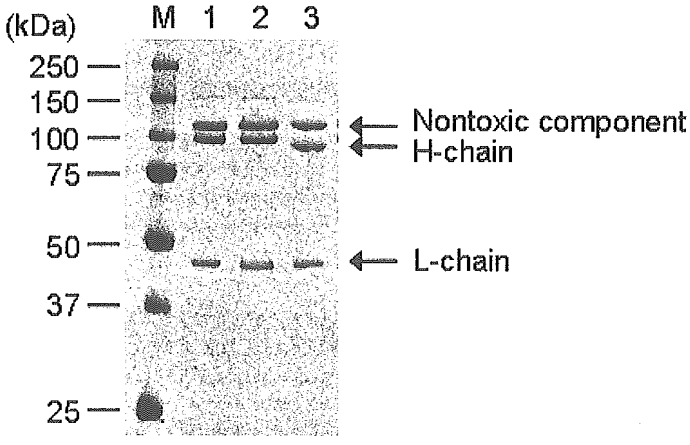

Fig. 5. SDS-PAGE profiles of M toxins of strains CB-19, 003-9, and 1873. Electrophoresis of each M toxin (2 μg) was performed on 10% gel in the presence of dithiothreitol. Lane 1: CB-19; Lane 2: 003-9; and Lane 3: 1873. M, molecular weight marker proteins (Precision Plus Protein All blue Standards, Bio-Rad, Hercules, CA); kDa, kilodalton.

analysis revealed that 003-9/BoNT consists of C/D mosaic toxin as well (27).

We did not find a significant difference in lethality to mice between type C and C/D mosaic toxins. However, the C/D mosaic toxin was more lethal than the type C toxin to chickens. The results also support the notion that the C/D mosaic toxin, but not type C toxin, is related to avian botulism. The action of toxins has been proposed to involve a series of events, including receptor binding, translocation, and enzymatic action (26). Since there is an intrinsic molecular difference between the H_C of C/D mosaic and type C BoNTs, it is unlikely that they share the same binding site on the target cells, which may reflect their host specificity with regard to chickens. From the present observations, we could not obtain any direct clue regarding the relationship to the occurrence of avian botulism. However, the unique structure of C/D mosaic BoNT associated with avian botulism should contribute to the development of a vaccine and immunologic methods to devise a countermeasure for avian botulism (18).

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Intrabodies against the EVH1 domain of Wiskott–Aldrich syndrome protein inhibit T cell receptor signaling in transgenic mice T cells

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Keywords
cytosolic protein; functional knockdown; intrabody; T-cell receptor signaling; Wiskott–Aldrich syndrome protein (WASP)

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Intracellularly expressed antibodies (intrabodies) have been used to inhibit the function of various kinds of protein inside cells. However, problems with stability and functional expression of intrabodies in the cytosol remain unsolved. In this study, we show that single-chain variable fragment (scFv) intrabodies constructed with a heavy chain variable (V_H) leader signal sequence at the N-terminus were translocated from the endoplasmic reticulum into the cytosol of T lymphocytes and inhibited the function of the target molecule, Wiskott–Aldrich syndrome protein (WASP). WASP resides in the cytosol as a multifunctional adaptor molecule and mediates actin polymerization and interleukin (IL)-2 synthesis in the T-cell receptor (TCR) signaling pathway. It has been suggested that an EVH1 domain in the N-terminal region of WASP may participate in IL-2 synthesis. In transgenic mice expressing anti-EVH1 scFvs derived from hybridoma cells producing WASP-EVH1 mAbs, a large number of scFvs in the cytosol and binding between anti-EVH1 scFvs and native WASP in T cells were detected by immunoprecipitation analysis. Furthermore, impairment of the proliferative response and IL-2 production induced by TCR stimulation which did not affect TCR capping was demonstrated in the scFv transgenic T cells. We previously described the same T-cell defects in WASP transgenic mice overexpressing the EVH1 domain. These results indicate that the EVH1 intrabodies inhibit only the EVH1 domain function that regulates IL-2 synthesis signaling without affecting the overall domain structure of WASP. The novel procedure presented here is a valuable tool for *in vivo* functional analysis of cytosolic proteins.

Intracellular antibodies (intrabodies) may be useful tools for not only clinical applications such as viral neutralization and cancer therapy but also functional analysis of proteins inside cells. A variety of intrabody formats have been used. Single-chain variable fragments (scFvs) consist of one heavy chain variable region (V_H) linked through a flexible peptide spacer, usually a repeated motif of 3 × GGGGS, to one light

Abbreviations
BrdU, 5-bromo-2′-deoxyuridine ER, endoplasmic reticulum; intrabody, intracellular expressed antibody; EVH1, enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) homology 1; FITC, fluorescein isothiocyanate; GST, glutathione S-transferase; IL, interleukin; scFv, single-chain variable fragment; TCR, T cell receptor; VH, heavy chain variable; VL, light chain variable; WASP, Wiskott–Aldrich syndrome protein; WIP, WASP-interacting protein.

chain variable (V_L). They are able to fold and retain the antigen-binding specificity and affinity of the parental antibody [1,2]. scFvs are expressed more easily than whole antibodies assembled with heavy and light chains by disulfide bonds. In general, the antibody fragments for assembling the scFvs are isolated from either antibody phage display libraries [3] or well-characterized hybridoma producing mAbs. Although screening of phage libraries allows the selection of antibody fragments directed against a variety of antigens, the screened antibody fragments often show low or intermediate affinity for the antigen. Therefore, large-scale libraries and extensive screening are required for the selection of the antigen-specific antibody fragments. On the other hand, the antibody fragments isolated from hybridomas have high affinity and specificity for the target molecules. However, the cloning of heavy and light chain variable regions by RT-PCR can be difficult because of the presence of nonspecific variable region transcripts produced by myeloma cells that are fused to the antibody-producing cells.

In functional proteomics, comprehensive protein analyses have been demonstrated [4]. However, the development of a new procedure for domain analysis of protein is necessary. Gene knock-out technologies that rely on developing a phenotype from null mutation of the gene in embryonic stem cells are powerful tools for understanding gene function. Recently, RNA interference (RNAi) which can eliminate specific mRNA and lead to gene silencing has been developed [5]. However, these gene knock-out and silencing techniques cannot be used to analyze domain structures and functions and post-translationally modified protein functions. Dominant negative gene knock-out procedures succeed in inhibiting the targeted domain functions of proteins, but not in all cases.

Antibodies have been used for various purposes for a long time. For example, they have been used as reagents for Western blotting, immunostaining, immunoprecipitation and blocking of protein function. Therefore, if intrabodies retain their specificity and high-affinity binding properties, they may be useful tools for inhibition of protein function inside the cell. In fact, much attention has been paid to intrabodies for clinical applications. The functional knockdown of target proteins, such as HIV gp120, chemokine receptor, growth factor receptors, MHC class I, Ras oncogene, p53 tumor suppressor, and protein kinases has been demonstrated [6–12]. If the target proteins are synthesized and processed in the endoplasmic reticulum (ER), scFvs are expressed with the signal peptide at the N-terminus of V_H and V_L with the ER retention signal KDEL (Lys-Asp-Glu-Leu) at the C-terminus. Folded scFvs can bind to the target proteins on the lumen side and inhibit transport of target proteins in the process of functional maturation [6,8]. If the targets are cytosolic proteins, scFvs without the signal peptide are used for expression in the cytosol. However, expression levels of scFvs are low in the cytosol, and binding of scFv intrabodies to target molecules is difficult to detect [13]. A small quantity of intrabodies in the cytosol may explain the low translational efficiency and low stability of intrabodies in the cytosol.

Wiskott–Aldrich syndrome protein (WASP), the causal gene product of the X-linked immunodeficiency (WAS) [14,15], participates in TCR signaling as a cytosolic adaptor molecule [16–18]. It is well known that TCR stimulation activates various signaling cascades accompanied by recruitment of adaptor molecules, protein kinases and regulatory molecules into the membrane-receptor complexes, resulting in the correct initiation and amplification of the signaling reaction. WASP is an adaptor molecule containing multiple domains: for example, a GTPase-binding domain, which is thought to interact with Cdc42, and a proline-rich region, which interacts with the Src homology 3 domain of the adaptor Nck, Grb2 and several kinases [19–22]. Furthermore, WASP is also associated with the actin-related protein (Arp2/3) complex through its C-terminal region. The association of WASP and the Arp2/3 complex activates the actin nucleation activity of the Arp2/3 complex [23].

To investigate further the function of the WASP-EVH1 domain in the TCR signaling pathway, we developed transgenic (Tg) mice that express intrabodies that specifically bind to the WASP-EVH1 domain. The cDNA fragments that encode variable regions of heavy and light chains were isolated from two established hybridomas producing WASP-EVH1-specific mAbs. We constructed several scFvs consisting of V_H and V_L regions with/without the V_H leader sequence at the N-terminal and with/without the $C_L(\kappa)$ region behind the V_L region. None of the constructs contained the KDEL sequence at the C-terminus. We compared the quantity of scFv intrabodies and assessed their binding activity to the WASP-EVH1 domain in the scFv gene-transfected T cells. Finally, we succeeded in expressing the functional scFv intrabodies in the cytosol and precisely knocking down the targeted protein domain in scFv transgenic mice.

Results

Construction of anti-WASP-EVH1 scFvs

To assess the binding activity to native WASP in T cells, mAb clones (17, 18 and 21) were confirmed

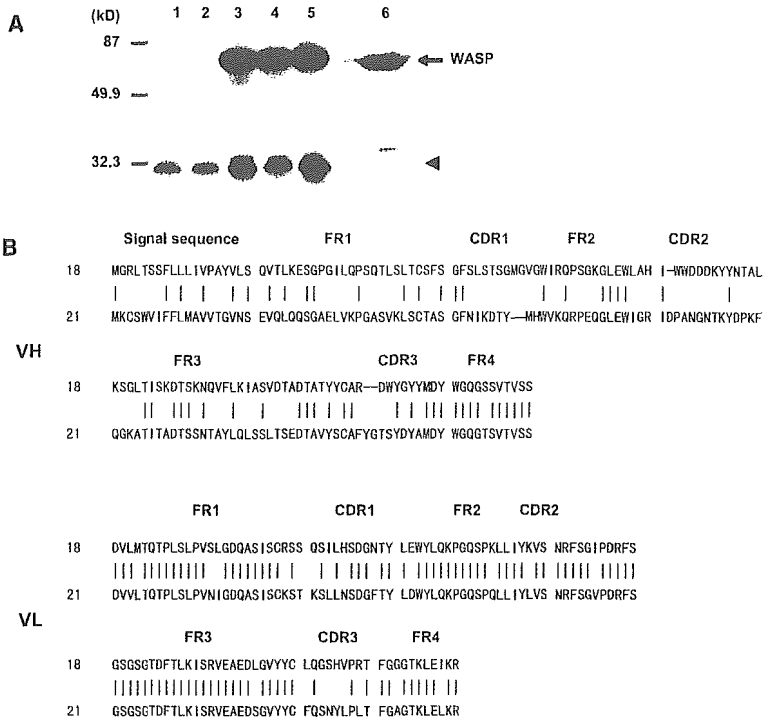

Fig. 1. Selection of WASP EVH1 mAbs for assembling scFvs and aligned amino-acid sequences of the V_H and V_L regions. (A) Immunoprecipitation of T cell lysates with WASP EVH1 mAbs produced by established hybridomas. T cell lysates were immunoprecipitated with 5 $\mu g \cdot mL^{-1}$ control mouse IgG (lane 1), clone 17 (lane 2), clone 18 (lane 3), clone 21 (lane 4) or commercially available WASP mAb (lane 5) and analyzed by Western blotting with WASP polyclonal antibody. Control T cell lysates were loaded in lane 6. The 30-kDa bands (arrowhead) indicated secondary antibody cross-reactive nonspecific proteins. (B) Comparison of deduced amino-acid sequences of the V_H and V_L fragments derived from WASP EVH1 mAbs 18 and 21. Shared amino acids are indicated by bars. Leader signal sequences and three complementarity-determining regions are shown in gray boxes. Four framework regions (FR) are marked above the sequence.

by immunoprecipitaion. Clones 18 and 21 were able to bind to the native form of WASP expressed in T cells, but clone 17 was not able to immunoprecipitate native WASP (Fig. 1A). On the basis of this result, clones 18 and 21 were selected for construction of scFv intrabodies. For the design of primers for PCR amplification of cDNA that encodes subtype-specific V_H and V_L regions, mAbs were checked by an isotyping test. Clones 18 and 21 were classified as IgG3/κ and IgG2b/κ, respectively. The appropriate cDNA fragments of the V_H and V_L regions were then generated by RT-PCR. A comparison of the V_H and V_L amino-acid sequences of clones 18 and 21 is shown in Fig. 1B. All of the V_H regions and the complementarity-determining region 3 of the V_L regions differed strongly between the two clones.

Generation of scFv from hybridomas was achieved by well-established molecular engineering methods. The four-step PCR using appropriate primers allowed amplification and assembly of the V_H and V_L regions (Fig. 2A). To investigate the stability of scFvs, we designed several scFv constructs with and without the N-terminal leader signal sequence of the V_H region and with and without the $C_L(\kappa)$ region following the V_L region, which are described as HL, SHL, HL-CL and SHL-CL in Fig. 2B.

Expression of scFv intrabodies and binding to WASP

In all scFv gene-transfected T cells, expression of scFv intrabodies was detected by Western blot analysis. However, scFvs containing the V_H signal peptide sequence and C_L region (SHL or SHL-CL) were highly expressed in T cells (Fig. 3A). These results strongly suggest that the addition of the V_H signal peptide sequence and $C_L(\kappa)$ region to scFvs increases the stability of the scFv intrabodies in T cells.

An *in vitro* binding assay was performed using glutathione S-transferase (GST) pull-down to detect the binding activity of anti-WASP scFvs. Constructs containing the V_H signal peptide sequence, 18SHL/SHL-CL and 21SHL/SHL-CL, were able to bind to GST-WASP15 (see Experimental procedures for definition of WASP15), whereas no binding activity of scFvs that did not contain the signal peptide sequence of the V_H region was detected (Fig. 3B). Although the expression levels of 18SHL/SHL-CL and 21SHL/SHL-CL were almost the same in the scFv gene-transfected T cells, 21SHL/SHL-CL bound more strongly to GST-WASP15 than 18SHL/SHL-CL (Fig. 3A,B). Furthermore, tq examine the interaction *in vivo* between scFv intrabodies and the target molecule, WASP, scFv gene-transfected T cells were lysed

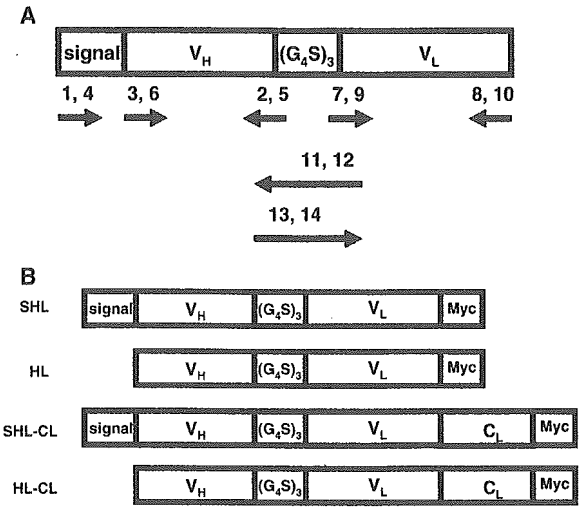

Fig. 2. Constructions of anti-WASP EVH1 scFvs. (A) Cloning of variable region of immunoglobulin heavy and light chains from hybridoma cells producing WASP EVH1 mAb. The arrows represent the following primers used to amplify the antibody fragments: primer 1, 5′-CACCCAAGCTTGCCACCATGGGCAGACTTACTTCTTCATTC-3′; primer 2, 5′-**CAGAACCACCACCCCC**TGAGGAGACGGTGACTGAGG ATCC-3′; primer 3, 5′-CACCCAAGCTTGCCACCATGCAGGTTACTCT GAAAGAGTC-3′; primer 4, 5′-CACCCAAGCTTGCCACCATGAAATG CAGCTGGGTTATCTTC-3′; primer 5, 5′-**CAGAACCACCACCCCC**TG AGGAGACGGTGACTGAGGTTCC-3′; primer 6, 5′-CACCCAAGCTT GCCACCATGGAGGTTCAGCTGCAGCAGTCTG-3′; primer 7, 5′-**GGT GGAGGAGGTTCT**GATGTTTTGATGACCCAAACTCCAC-3′; primer 8, 5′-CGAATGCGGCCGCCCGTTTGATTTCCAGCTTGGTGC-3′; primer 9, 5′-**GGTGGAGGAGGTTCTGATGTTGTTCT**GACCCAAACTCCACTC-3′; primer 10, 5′-CGAATGCGGCCGCCCGTTTCAGCTCCAGCTTGGTCC-3′; primer 11, 5′-TCAAAACATC**AGAACCTCCTCCACCGGATCCTCCAC CTCCAGAACCACCACCCCC**-3′; primer 12, 5′-GAACAACATC**AGAA CCTCCTCACCGGATCCTCCACCTCCAGAACCACCACCCCC**-3′; primer 13, 5′-CGTCTCCTCA**GGGGGTGGTGGTTCTGGAGGTGGAG GATCCGGTGGAGGAGGTTCT**-3′; primer 14, 5′-CGTCTCCTCA **GGGGGTGGTGGTTCTGGAGGTGGAGGATCCGGTGGAGGAGG TTCT**-3′. In all primers, underlined sequences indicate restriction site of *Hin*dIII and *Not*I, and bold letters indicate full or part of the $(Gly_4\text{-}Ser)_3$ linker sequence. (B) Schematic representation of the four scFv formats (SHL, HL, SHL-CL, and HL-CL). Shown are the leader signal sequence, V_H region, polypeptide linker $(G_4S)_3$, V_L region, light chain constant $[C_L(\kappa)]$ region and Myc tag sequence.

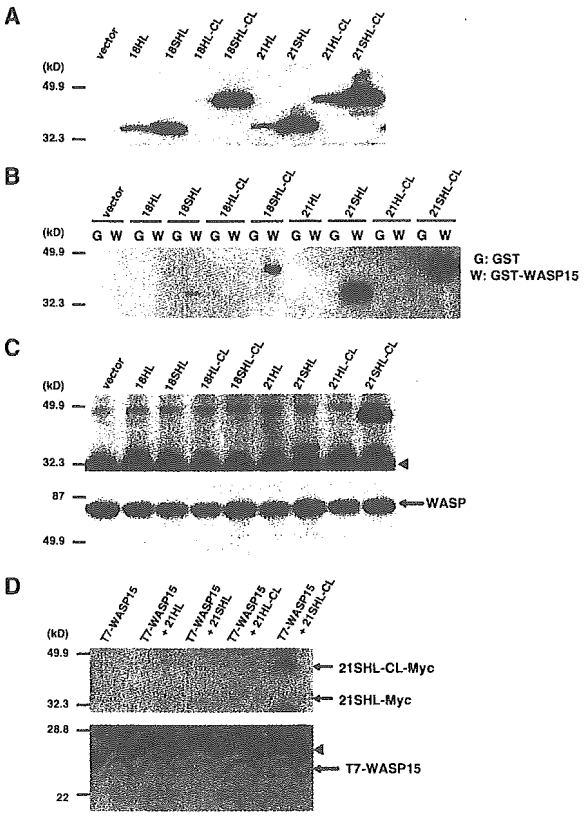

Fig. 3. Expression of anti-WASP scFvs and detection of their binding activity to WASP in T cells. (A) Western blot analysis of protein extracts of anti-WASP scFv DNA-transfected T cells. The immunoblot was probed with Myc tag mAb. (B) *In vitro* binding assay using GST pull-down. All anti-WASP scFv DNA-transfected T cells were lysed and incubated with GST (G) or GST-WASP15 (W) fusion protein noncovalently bound to glutathione–Sepharose beads. Bound proteins were analyzed by Western blotting with Myc tag mAb. (C) *In vivo* association between scFvs and WASP. All scFv DNA-transfected cell lysates were immunoprecipitated with WASP mAb and analyzed by Western blotting with Myc tag mAb (top panel) or WASP mAb (bottom panel). (D) EVH1 domain-specific binding of scFv T7-WASP15 and scFv DNA cotransfected cell lysates were immunoprecipitated with biotinylated T7 tag mAb. Immunocomplexes were recovered by on streptavidin–agarose and analyzed by Western blotting with Myc tag mAb (top panel) or T7 tag mAb (bottom panel). Arrowheads indicate secondary antibody cross-reactive nonspecific proteins.

and immunoprecipitated with WASP mAb. A strong interaction between WASP and 21SHL/SHL-CL scFvs was detected by Western blot analysis with Myc tag antibody, whereas 18SHL/SHL-CL scFvs and other scFvs were not able to associate with native WASP (Fig. 3C). The binding specificity for the WASP EVH1 domain was demonstrated by *in vivo* interaction between T7-tagged WASP15 and 21SHL/SHL-CL scFvs (Fig. 3D). These results suggest that 21SHL and 21SHL-CL are stably expressed as intrabodies with domain-specific binding capabilities, and are able to associate with native WASP in T cells.

To detect cleavage of the V_H signal peptide sequence from the N-terminal scFv-V_H region, the N-terminal amino-acid sequence of scFv 21SHL-CL expressed in T cells was determined. Unfortunately, we could not detect the N-terminal sequence by the well-established Edman method because the N-terminal amino-acid residue was blocked. Moreover, we could not detect a

V_H signal peptide sequence by MS analysis of 21SHL-CL digested with lysyl endopeptidase (data not shown). These results suggest that the V_H signal peptide sequence was cleaved from the N-terminal V_H region. The culture supernatant of 21SHL-CL scFv-expressed T cells was examined but the scFv could not be detected (data not shown). These results suggest that, even if scFvs are expressed with a signal sequence, they do not enter the secretory pathway.

Generation of anti-WASP scFv transgenic mice

scFv 21SHL and 21SHL-CL vector DNAs were chosen as the transgenes for development of transgenic mice with the functional knockdown WASP-EVH1 domain.

High expression of 21SHL and 21SHL-CL was detected in T and B cells from the spleens of the 21SHL/21SHL-CL scFv transgenic mice (Fig. 4A). Eight 21SHL transgenic founders and 10 21SHL-CL transgenic founders carrying the scFv intrabody expression vectors were obtained. In four of eight 21SHL lines and five of 10 21SHL-CL lines, the same levels of expression of 21SHL and 21SHL-CL were

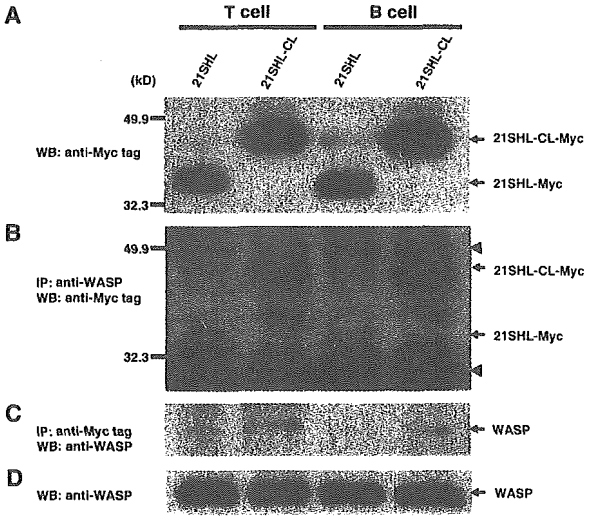

Fig. 4. Expression of anti-WASP scFvs and *in vivo* interaction between scFvs and WASP in scFv transgenic mice T and B cells. (A) Western blot analysis of protein extracts of T and B cells from the spleens of the 21SHL and 21SHL-CL scFv transgenic mice. The immunoblot was probed with Myc tag mAb. (B, C) *In vivo* association between scFvs and WASP. The scFv 21SHL and 21SHL-CL transgenic T and B cell lysates were immunoprecipitated with WASP mAb and Myc tag mAb and analyzed by Western blotting with Myc tag mAb and WASP mAb. Arrowheads indicated secondary antibody cross-reactive nonspecific proteins. (D) Both scFv transgenic mice T and B cell lysates were analyzed by Western blotting with WASP antibody.

detected (data not shown). Furthermore, T and B cells from the spleens of both scFv transgenic mice were solubilized with 1% digitonin buffer and immunoprecipitated with WASP mAb and Myc tag mAb to examine the *in vivo* interaction between scFvs and endogenous WASP. Binding of intracellular scFvs and WASP was detected in both T and B cells from scFv transgenic spleens by immunoprecipitation (Fig. 4B–D).

Impaired antigen receptor-induced proliferation in anti-WASP scFv transgenic T cells, but not B cells

To assess the effects of the anti-WASP scFvs 21SHL and 21SHL-CL on T-cell function, the proliferative response to stimulation with CD3ε antibody (2c11) was examined. Compared with the wild-type, T cells from 21SHL transgenic mice and 21SHL-CL transgenic mice were impaired in their proliferative response to CD3ε antibody stimulation to the same extent as in WASP15 transgenic T cells [24] (Fig. 5A). These findings indicate that the function of the WASP N-terminal EVH1 domain is blocked by scFv 21SHL and 21SHL-CL intrabodies in the T cells. In contrast with T cells, proliferative responses to antigen receptor stimulation with anti-IgM Ab $F(ab')_2$ or CD40 antibody were normal in the scFv transgenic B cells (Fig. 5B). Therefore, the EVH1 domain of WASP is not functional, at least in the Ag receptor-induced proliferative response of B cells.

T cells from the other three 21SHL transgenic lines and the other four 21SHL-CL transgenic lines were also impaired in their proliferative response to stimulation with CD3ε antibody (data not shown), confirming that there were no problems in the integration site of the transgene.

Lymphoid development in anti-WASP scFv transgenic mice

T-cell development in the spleen can be followed by examining the expression patterns of the CD4 and CD8 surface antigens. The population of mature single-positive thymocytes (either $CD4^+CD8^-$ or $CD4^-CD8^+$) was almost the same in wild-type, 21SHL transgenic, and 21SHL-CL transgenic mice (Fig. 5C). Likewise the expression pattern of CD3 was nearly the same. Furthermore, the percentages of splenic T and B lineage cell populations were normal (Fig. 5C). In addition, T lineage cell populations in the thymus and B lineage cell populations in the bone marrow were almost the same for wild-type, 21SHL transgenic and 21SHL-CL transgenic mice (Fig. 5D,E). These results

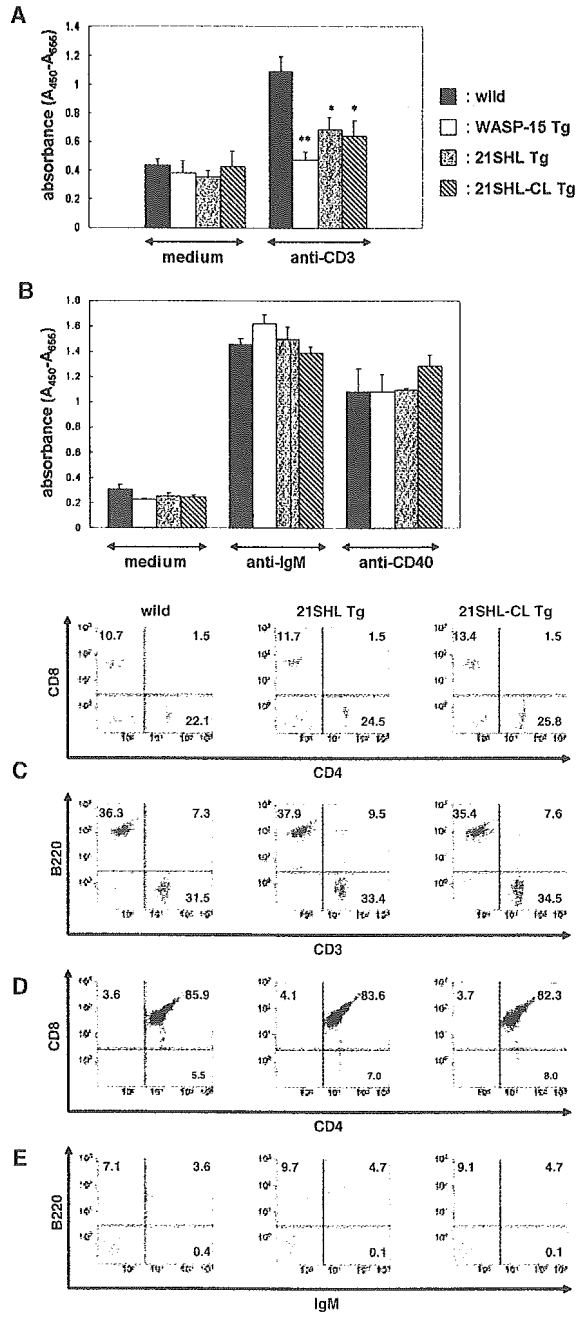

Fig. 5. Antigen receptor-induced proliferation in anti-WASP scFv transgenic T and B cells, and lymphoid development in anti-WASP scFv transgenic mice. (A) T-cell proliferation. Splenic T cells from anti-WASP scFv 21SHL transgenic, 21SHL-CL transgenic, WASP15 transgenic and wild-type mice were cultured in medium alone or in the presence of CD3ε antibody. (B) B-cell proliferation. Splenic B cells from anti-WASP scFv 21SHL transgenic, 21SHL-CL transgenic, WASP15 transgenic and wild-type mice were cultured in medium alone or in the presence of IgM antibody $F(ab')_2$ or CD40 antibody. Each stimulation was performed in the presence of exogenous IL-4. In each experiment, cells were cultured for 48 h, then 10 μM BrdU was added to the T and B-cell cultures. The cells were reincubated for an additional 16 h, and BrdU incorporation was quantified by ELISA. Values represent means ± SE of triplicate cultures and are representative of three independent experiments. Statistical significance is indicated by *($P < 0.05$) and **($P < 0.005$). (C)–(E) FACS analyses of lymphocytes from wild-type, anti-WASP scFv 21SHL transgenic and 21SHL-CL transgenic mice. Two-color flow cytometric analyses were performed on spleen (C), thymus (D) and bone marrow (E). Percentages of representative lymphoid populations are noted. The results shown are representative of at least three male mice for each analysis at the age of 8 weeks.

indicate that anti-WASP scFvs do not have a marked effect on lymphocyte development.

Impaired interleukin (IL)-2 production induced by TCR stimulation, but not antigen receptor capping

To assess whether the 21SHL and 21SHL-CL scFvs affect IL-2 production induced by TCR stimulation, purified T cells from spleens of wild-type, WASP15 transgenic, 21SHL transgenic and 21SHL-CL transgenic mice were stimulated with immobilized CD3ε antibody and IL-2 in the culture supernatant and determined by ELISA. T cells expressing 21SHL and 21SHL-CL scFvs were impaired in IL-2 production induced by TCR stimulation, whereas the defect in IL-2 production of scFv transgenic T cells was slight compared with the WASP15 transgenic T cells (Fig. 6A).

In addtion, purified T cells were incubated *in vitro* with fluorescein isothiocyanate (FITC)-conjugated CD3ε antibody at either 37 °C or 4 °C (stimulation or nonstimulation) to assess whether the 21SHL and 21SHL-CL scFvs affect TCR-induced capping. The rate of antigen-receptor capping of T cells was the same in all the mice (Fig. 6B). These results indicate that the anti-WASP scFvs 21SHL and 21SHL-CL inhibit the signaling cascade of IL-2 production via TCR stimulation without affecting the regulation of the cytoskeleton, including antigen-receptor capping. These findings strongly indicate that IL-2 synthesis is mediated directly by the WASP EVH1 domain and not by secondary events resulting from WASP-mediated actin cytoskeletal rearrangements induced by TCR signaling.

Subcellular localization of anti-WASP scFvs

To examine the subcellular localization of anti-WASP scFvs 21SHL and 21SHL-CL in T cells, cell extracts of their scFv-transgenic T cells were fractionated into the subcellular compartments, cytosolic proteins and

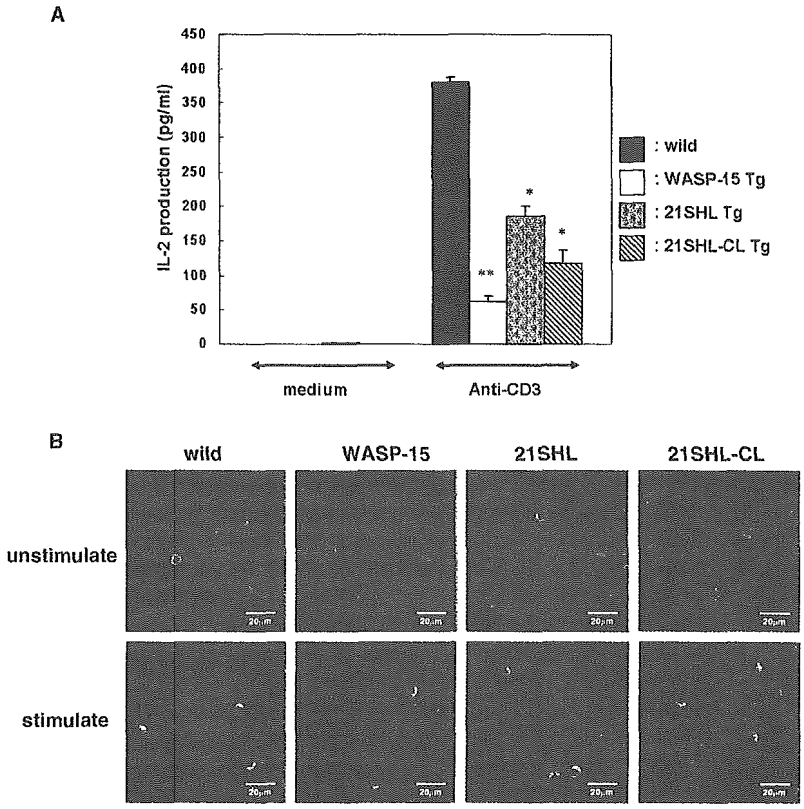

Fig. 6. IL-2 production was impaired, but not antigen receptor capping induced by TCR stimulation. (A) Splenic T cells from anti-WASP scFv 21SHL transgenic, 21SHL-CL transgenic, WASP15 transgenic and wild-type mice were cultured in medium alone or in the presence of anti-CD3ε Ab. Each cell culture supernatant was collected at 24 h. IL-2 in the supernatant was quantified by ELISA. Values are mean ± SE from triplicate cultures and are representative of three independent experiments. Statistical significance is indicated by *($P < 0.005$) and **($P < 0.001$). (B) Splenic T cells from anti-WASP scFv 21SHL transgenic, 21SHL-CL transgenic, WASP15 transgenic and wild-type mice were incubated with FITC-conjugated CD3ε antibody at either 4 °C or 37 °C for 30 min. The treated cells were placed on polyethylenimine coated eight-well tissue culture glass slides, fixed, analyzed and photographed at × 100 using confocal microscopy. The rate of capping of unstimulated and stimulated T cells was determined by counting the number of caps in ≈200 cells/experiment. The wild-type and transgenic mice used for these experiments were 8 weeks old.

membrane/membrane organelles. In general, the scFv intrabodies (V_H–linker–V_L format) with heavy chain signal peptide sequences cross the rough ER membrane and enter the secretory pathway through the *trans*-Golgi network. However, equivalent amounts of scFv 21SHL were detected in both the cytosol and membrane fractions, and most of the scFv 21SHL-CL was detected in the cytosol fraction in anti-WASP scFv transgenic T cells (Fig. 7A). To confirm the presence of cross-contamination in both fractions of scFv 21SHL-CL transgenic T cells, each fraction was examined by Western blotting with WASP antibody and Ribophorin I antibody specifically expressed in the cytosolic and membrane fractions, respectively. These results show that neither fraction was cross-contaminated (Fig. 7A).

When scFv intrabodies were expressed in NIH-3T3 fibroblastic cells, the scFvs were localized in the subcellular compartments. NIH-3T3 cells were transfected with scFv 21SHL-CL (with leader signal sequence) or 21HL-CL (without leader signal sequence) genes and then their subcellular fractions were subjected to Western blotting with Myc tag antibody. The majority of the intrabodies expressed without signal sequence were detected in the cytosol, whereas most of the intrabodies expressed with the signal sequence were detected in the membrane fraction (Fig. 7B). These results indicate that the post-translational processing of ER-coupled protein synthesis must be different among cell types such as lymphocytes and fibroblasts.

On immunostaining, colocalization of 21SHL-CL scFv and endogenous WASP was observed in the cytosol of the scFv DNA transfected T cells (Fig. 7C). Again these results indicate that scFv intrabodies expressed with the V_H signal peptide sequence are localized in the cytosol of T cells. Taken together, the results strongly suggest that scFv intrabodies synthesized in the ER are released from the ER membrane into the cytosol by retro-translocation in lymphocytes including T cells [25].

In general, when proteins synthesized in the ER are misfolded or incompletely assembled into oligomeric forms, they are transferred from the lumen of the ER into the cytosol, so-called retro-translocation. In the cytosol, the retro-translocated proteins are polyubiquitinated and degraded by proteasomal proteolysis [26–29]. Our results suggest that the WASP scFv intrabodies expressed with the V_H signal sequence are translocated across the ER membrane into the cytosol without degradation. The cell lysates or immunopre-