Interaction between the two subdomains of the C-terminal part of the botulinum neurotoxin A is essential for the generation of protective antibodies ☆

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Abstract The botulinum neurotoxin A C-terminal fragment (Hc), which mediates the binding of the toxin to neuronal cell surface receptors, comprises two subdomains, Hc-N (amino acids 873–1095) and Hc-C (amino acids 1096–1296). In order to define the minimal fragment of Hc carrying protective antigenic properties, Hc, Hc-N and Hc-C have been produced as recombinant proteins in *Escherichia coli*, and have been tested for their antigenicity in mouse protection assays. Hc, Hc-N and Hc-C induced similar antibody levels as shown by ELISA. However, a single immunization with Hc (10 μg) fully protected mice challenged with 10^3 mouse lethal dose 50 of toxin, whereas Hc-N, Hc-C, or Hc-N plus Hc-C did not give any protection. Triple immunizations with Hc-N or Hc-C were necessary to induce a higher level of protection. Circular dichroism and fluorescence studies showed that the isolated subdomains were folded and stable. However, an intense near-UV dichroic signal was only observed in the Hc spectrum, revealing a highly structured interface between both subdomains. Taken together, the results show that the generation of protective antibodies requires the whole Hc domain and especially the native structure of the interfacial region between Hc-N and Hc-C.

Keywords: Botulinum neurotoxin A C-terminal fragment; Protective antibodies; Antibodies

1. Introduction

Botulinum neurotoxin A (BoNT/A) is the most potent toxin among all the bacterial, animal, plant toxins and chemical compounds. It is responsible for severe food intoxication in humans and animals, called botulism. Based on its high level of activity, BoNT/A is considered as a potential weapon. In addition, BoNT/A is also a therapeutic agent, which is used in the treatment of numerous dystonias [1,2]. BoNT/A is produced as a single chain protein, which is poorly active, and it is proteolytically cleaved in an active di-chain form constituted of a light (about 50 kDa) and a heavy (about 100 kDa) chain linked by a disulfide bridge. The C-terminal half of the heavy chain, named fragment C (Hc), recognizes a surface receptor on neurons, which provokes the internalization of the whole toxin into endocytic vesicles. The N-terminal half of the heavy chain mediates the translocation of the light chain, which is the intracellular active domain, into the cytoplasm of the neuron. In the motor nerve ends and autonomic cholinergic junctions, BoNT/A cleaves SNAP25, which is one of the three SNARE proteins having a determinant role in neuroexocytosis. Therefore, BoNT/A blocks the Ca^{2+} induced release of acetylcholine and causes flaccid paralysis [3–5]. The crystal structure of BoNT/A has been solved and the functional domains correspond to distinct structures [6]. Hc (residues 873–1296) remains isolated from the rest of the molecule and comprises two subdomains corresponding to amino acids 873–1095 (Hc-N) and amino acids 1096–1296 (Hc-C). Hc-N and Hc-C are β-strand rich structures of almost identical sizes. Hc-N has two seven-stranded β-sheets, while Hc-C adopts a modified β-trefoil with a β-hairpin capping the base of the domain (Fig. 1). The two subdomains have possibly evolved from an ancestral binding domain [6,7]. The precise role of Hc-N and Hc-C in the binding to the cell surface receptor, which could involve a ganglioside and a membrane protein, has not been fully determined (review [5]).

Prevention of botulism can be efficiently achieved by vaccination, which generates neutralizing antibodies against botulinum neurotoxin. Botulinum vaccines are basically formalin-inactivated culture supernatants from *Clostridium botulinum* grown in fermentor or purified neurotoxin. This includes large-scale production of highly toxic material and inactivation by formaldehyde. To reduce the cost and increase safety conditions, subunit vaccines based on immunogenic non-toxic fragments of the neurotoxin have been developed. Such attempt has been first performed with tetanus toxin.

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Abbreviations: BoNT/A, botulinum neurotoxin A; CD, circular dichroism; Hc, botulinum neurotoxin A C-terminal fragment; Hc-N, N-terminal subdomain of Hc; Hc-C, C-terminal subdomain of Hc; MLD, mouse lethal dose

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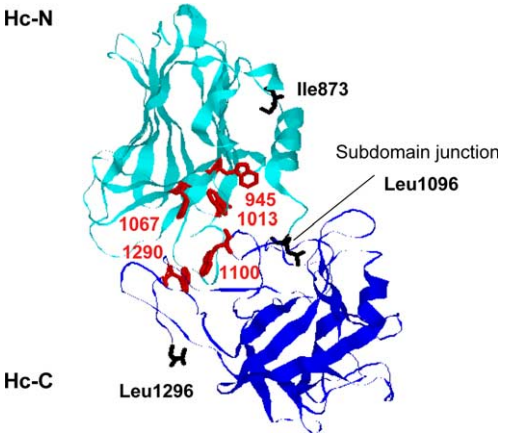

Fig. 1. Structure of the BoNT/A receptor binding domain (Hc) and the two subdomains Hc-N and Hc-C according to [6]. The five Trps located at the interface of the two subdomains are shown in red. The brackets indicate the interfacial region (IR). Residues 873, 1096 and 1296 label the termini residues of each subdomains.

Indeed, recombinant Hc from tetanus toxin was found to protect mouse challenged with active tetanus toxin [8]. Similar strategy was used with BoNT/A [9] and a new generation of botulinum vaccines has been designed on the basis of recombinant Hc of the various BoNT types [10]. Moreover, neutralizing antibodies constitute the only specific treatment of botulism. Monoclonal antibodies and F_{ab} fragments from animal polyclonal antibodies are more appropriate than serotherapy for a safe treatment. However, numerous attempts to produce high-affinity neutralizing monoclonal antibodies by immunizing mice with detoxified BoNT have been unsuccessful. Since Hc from BoNT/A elicits neutralizing antibodies [9], this domain has been used as immunogen and has permitted to generate mouse neutralizing monoclonal antibodies [11,12]. Therefore, recombinant immunogenic BoNT Hc domain proves to be an efficient antigen to generate neutralizing antibodies for immunotherapy and also an appropriate vaccine candidate. Since the Hc domain of BoNT/A contains two subdomains, we have investigated the potency of each subdomain to induce a protective response in mice, in order to define the minimal fragment of Hc carrying protective antigenic properties.

2. Materials and methods

2.1. Plasmids, production and purification of recombinant proteins

DNAs coding for Hc, Hc-N and Hc-C were PCR amplified from *C. botulinum* NCTC2916 whole DNA with primers P5 (5′-GGATCCAATATTATTAATACTTCTATATTGAATTT- AAG-3′) and P729 (5′-GTCGACTTACAGTGGCCTTTC- TCCCCA-3′) for Hc, P5 and P747, (5′-GTCGACCTGAATTTGATTGATTATCATATAAATC-3′) for Hc-N, and P748 (5′-GAATTCATTTTAAAAGACTTTTGGGGTGAT- TATTTAC-3′) and P729 for Hc-C. The primers add a *Bam*HI site at the 5′ part and a *Sal*I site at the 3′ part of the DNAs coding Hc and Hc-N, and *Eco*RI and *Sal*I sites for the DNA coding Hc-C. Amplified DNAs were cloned into pET28b (Novagen) and the recombinant plasmids were transformed into *Escherichia coli* strain BL21. DNA constructs were checked by DNA sequencing. The recombinant strains were grown in LB medium supplemented with kanamycin (50 μg/mL), at 25 °C until OD 0.8, induced with isopropyl-1-thio-β-D-galactopyranoside (0.5 mM IPTG) and grown at 25 °C for an additional 20 h. The bacterial cells were collected by centrifugation, suspended in 50 mM NaH_2PO_4, pH 8, 300 mM NaCl, and lyzed by sonication. The lysates were centrifuged and filtered on a 0.45 μm filter, and the six His-tag fusion proteins were purified on a cobalt column (Clontech) according to the recommendations of the manufacturer. Elution was done with 150 mM imidazole in the same buffer. Purified proteins were analyzed by 10% SDS–polyacrylamide gel electrophoresis (PAGE). For spectroscopy studies, the recombinant proteins were desalted on a Sephadex® G-25 Superfine Hitrap™ desalting column (Amersham Biosciences) against 5 mM sodium phosphate, pH 7.4.

2.2. Circular dichroism (CD) spectroscopy

The measurements were done at 22 and 37 °C with constant N_2 flushing as described previously [13,14]. Far-UV and near-UV CD spectra were measured in 1 and 10 mm path length quartz cells, respectively. The protein concentrations were greater than 10 μM (1 mL) for near-UV CD and ranging from 2 to 5 μM (200 μL) for far-UV acquisitions. The scans were recorded using a bandwidth of 2 nm and an integration time of 1 s at a scan rate of 0.5 nm/s. Each spectrum represents the average of 20 scans. Whatever the UV region investigated, the blank was 5 mM sodium phosphate, pH 7.4. The spectra were corrected for blank and then, a smoothing algorithm was applied with the minimum filter in the CD6 software (CDMax, filter 5). The mean residue ellipticity in far-UV, $[\theta]_F$ and the molar ellipticity in near-UV, $[\theta]_N$ were, respectively, calculated from the relations $[\theta]_F = (100 \times \theta_m)/(C \times l \times N)$ and $[\theta]_N = (100 \times \theta_m)/(C \times l)$, where θ_m is the measured ellipticity in degrees, C is the concentration in mole per liter, l is the path length of the cell in centimeter and N is the number of residues. The value 100 arises from the conversion of the concentration in mole per liter to decimole per cubic centimeter. The units of the mean residue ellipticity $[\theta]_F$ and that of the molar ellipticity $[\theta]_N$ are expressed in degrees square centimeter per decimole residue (deg cm^2/dmol res) and in degrees square centimeter per decimole (deg cm^2/dmol), respectively. The spectra acquired in the region ranging from 220 to 260 nm are reported in molar ellipticity $[\theta]_N$, since we attribute these bands to tertiary constraints on aromatic residues (see the main text for details).

2.3. Fluorescence spectroscopy

Fluorescence measurements were performed with a FP-750 spectrofluorimeter (Jasco, Tokyo, Japan) in a thermostated cell holder using a 1 cm path length quartz cell with constant stirring. The temperature of the samples was regulated by a thermostated bath and monitored using a digital thermometer. Bandwidths of 5 nm were used for both excitation and emission beams. The excitation wavelength was fixed at 295 nm where the contribution of tyrosine residues is negligible. The emission spectra were recorded from 300 to 400 nm at a scan rate of 60 nm/min. The proteins were diluted to 1 μM in 2 mL of 5 mM sodium phosphate, pH 7.4. Spectra were recorded 10 min after the sample had reached the target temperature. For each temperature studied, the protein spectra were corrected for blank and the maximal emission wavelength (λ_{max}) was calculated from the average of at least four measured emission spectra.

2.4. Mouse protection assays

Swiss male mice (20–25 g) were injected intraperitoneally with 10 μg of recombinant protein homogenized in 1 mg aluminum hydroxide (Alugel, Serva) as indicated. Two weeks after the final immunization, blood samples were taken for ELISA titration and the mice were challenged with 10^3 mouse lethal dose (MLD)50 of BoNT/A per mouse.

2.5. Rabbit immunizations

New Zealand female rabbits (about 3 kg) were injected in foot pads with 100 μg recombinant protein in 1 mL PBS homogenized with 1 mL Freund's complete adjuvant for the first immunization and then Freund's incomplete adjuvant for the following injections (4 and 6 weeks after the initial immunization). Blood samples were collected 10 days after the last injection.

2.6. Immunoblotting analysis

Recombinant proteins (1 μg) were separated by SDS–PAGE and transferred to a nitrocellulose membrane (Hybond-C, Amersham). The membranes were blocked with 5% skimmed milk in PBS and were incubated with rabbit antibodies (1/100 000) against Hc, Hc-N or Hc-C for 1 h at room temperature. The blots were then treated with horse-

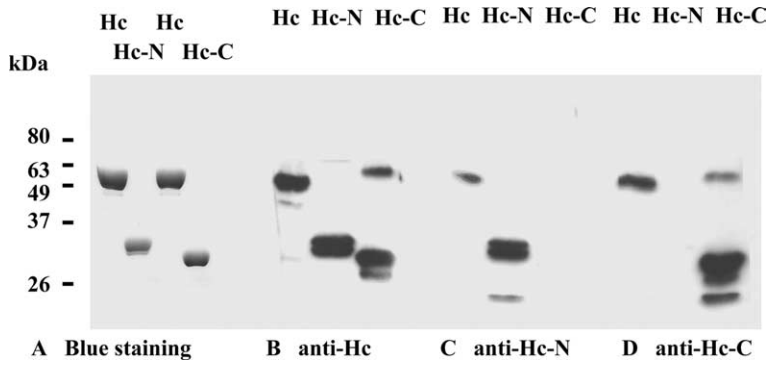

Fig. 2. SDS–PAGE and Coomassie blue staining of the recombinant proteins Hc, Hc-N and Hc-C (5 μg protein in each well) (A), and Western blotting with rabbit antiserum against whole Hc (B), Hc-N (C) and Hc-C (D). For Western blotting, 1 μg of recombinant protein was electrophoresed, transferred to nitrocellulose, and bound antibodies were visualized with horseradish peroxidase protein A and ECL kit.

radish peroxidase-conjugated protein A (Bio-Rad) and analyzed with the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech).

2.7. ELISA

Polyvinylchloride 96-well plates (Falcon) were coated with Hc (1 μg/mL in 15 mM Na_2CO_3, 36 mM $NaHCO_3$, pH 9.8, 100 μL/well) overnight at 4 °C and were blocked with PBS containing 0.1% gelatin (1 h, room temperature). The plates were washed four times with PBS containing 0.1% Tween 20 (Sigma) (PBST) and serial twofold dilutions in PBST starting at 1:200 of mouse serum samples were then added (100 μL/well). The plates were incubated 3 h at 37 °C and washed four times. Peroxidase anti-mouse immunoglobulin conjugate (Amersham Biotech; 1:2000; 100 μL/well) was added and the plates were incubated 1 h at 37 °C and washed. For IgG and IgM assays, peroxidase anti-mouse IgG and IgM immunoglobulins (Sigma A4416 and A8786) were used at 1:5000 and 1:200 dilution, respectively. The revelation was done with orthophenylenediamine (OPD, Sigma) and H_2O_2 and the reaction was stopped with 3 M HCl (50 μL/well). The plates were read with a microplate reader (490 nm).

2.8. Neutralizing antibodies

Neutralizing antibodies against BoNT/A were determined by the mouse test. Mixtures of serial dilutions of sera in phosphate buffer (50 mM Na_2HPO_4, pH 6.5) containing 1% gelatin with BoNT/A (10 MLD) in 1 mL final volume were incubated 30 min at room temperature and injected intraperitoneally in 20–22 g Swiss mice. The mice were observed for death until a period of 4 days. Results were expressed as inverses of the highest serum dilutions, which neutralize 10 MLD of BoNT/A.

3. Results

3.1. Recombinant Hc, Hc-N and Hc-C

Production and purification of the recombinant domains of BoNT/A were performed as described in Section 2. We found that the production of Hc and the two subdomains Hc-N and Hc-C in fusion with a six His-tag provided by the vector pET28 was increased when the recombinant strains were grown at low temperature (25 °C) and induced with a low concentration of IPTG (0.5 mM). The yields of production were approximately 10 mg of purified protein per liter of culture. The recombinant proteins were mostly recovered in the soluble fraction of the lysates and were purified on metal affinity columns. As shown in Fig. 2, purified Hc, Hc-N and Hc-C migrated at the expected size and were recognized by polyclonal antibodies raised against the whole Hc domain.

Cross immunogenicity between the two subdomains was tested by immunoblotting with rabbit serum raised against Hc-N and Hc-C. Antibodies against Hc-N or Hc-C recognized the corresponding recombinant proteins, respectively, and no cross-reaction was observed between the two subdomains (Fig. 2). Antibodies against Hc-C detected in addition to Hc-C migrating at the expected size, a 60 kDa band, which probably corresponded to partial protein aggregation.

3.2. Circular dichroism and fluorescence analysis

Fig. 3A shows the far-UV CD spectra of the three proteins. A positive band at 195 nm and a broad negative signal from 210 to 220 nm characterize the spectrum in the far-UV region of the isolated Hc-N fragment. These features correspond to a

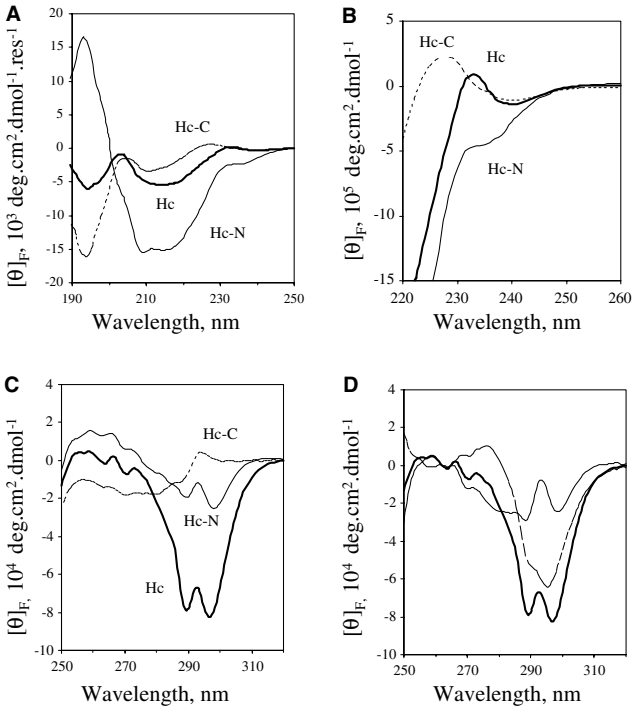

Fig. 3. CD spectra of the recombinant proteins in the far-UV (A), between 220 and 260 nm (B) and in the near-UV (C). Hc (bold line), Hc-N (thin line) and Hc-C (dotted line). In (D), the near-UV CD spectrum of Hc (bold line) is compared with the sum of the near-UV CD spectra of Hc-N and Hc-C (thin line). The difference between the two signals is shown as a dotted line.

typical signature of β-sheets. Thus, the isolated subdomain Hc-N was folded and its secondary structure content agreed with the X-ray structure of Hc-N within the whole domain, composed of 14 β-strands organized in two β-sheets and one α-helix [6] (Fig. 1). The absence of the negative band at 222 nm, characteristic of α-structures, can be ascribed to the low α-helix content (7%) found in the Hc-N subdomain.

The CD spectra of Hc and Hc-C showed a negative signal at 195 nm, a relatively weak positive band around 204 nm and a wide negative band between 210 and 220 nm characteristic of β-structures. The strong negative signal at 195 nm found in Hc-C suggests the presence of flexible regions, which could correspond to the loops of the Hc-C subdomain (Fig. 1). The rest of both spectra exhibit low intensities. Proteins mainly devoid of α-helices, like Hc (5%) and Hc-C (2.9%) [6], give low signals [15]. Moreover, CD spectroscopy is known to be poorly sensitive to β-structures. Hence, for proteins with few β-structures like Hc-C, which contains 20% of β-sheet [6], the CD spectrum is weak.

Overall, these results indicate the presence of β-structures in all three proteins, although positive and negative contributions from other secondary structure motifs or aromatic residues cannot be excluded.

The CD spectra of Hc and Hc-C exhibited positive bands at 233 and 228 nm, respectively (Fig. 3A and B). These bands could arise from rotational constraints on aromatic side chains, as described for other proteins [16–19]. At these wavelengths, such signals may imply different transitions from the aromatic rings [20]: the L_a transition of tyrosine and the B_b band of Trp, located at 229 and 225 nm, respectively, are good candidates. These positive bands most likely reflected the chiral activity of the same aromatic residue(s). The maximum wavelength of the Hc band was probably red-shifted as compared to the one of Hc-C (Fig. 3B) due to the relatively more elevated dichroic contribution in this region of the Hc polypeptide backbone to the resulting signal as compared to Hc-C [16]. Together, the results suggest that Hc-C preserved this element of tertiary structure when isolated or within the entire Hc domain.

The near-UV CD spectrum of the Hc domain (Fig. 3C) showed two weak negative bands at 264 and 270 nm, a broad and strong signal between 280 and 310 nm, including two negative bands at 290 and 297 nm with a positive band at 293 nm in-between. The first two fine bands may arise from vibronic transitions of phenylalanines. The fine bands above 290 nm could be assigned to L_b transitions of Trps [20]. The near-UV CD spectrum of the Hc-N subdomain displayed similar fine bands, but the strong negative signal ranging from 280 to 310 nm in the CD spectrum of Hc was not observed. The CD spectrum of Hc-C was essentially composed of two features: a broad negative band between 260 and 290 nm and a positive signal at 294 nm. These results indicate that several aromatic side chains in the isolated subdomains were buried in chiral environments, providing direct evidence that these isolated subdomains had a tertiary structure.

Nevertheless, the sum of the Hc-N and Hc-C near-UV CD spectra did not reflect the spectrum of Hc (Fig. 3C and D). Although the fine bands above 290 nm seemed preserved, the strong negative signal between 280 and 310 nm lacked. Such signal was unusually intense and probably involved the participation of several Trps with high chiral activities. We suggest that this band could arise from the tight packing of some or all five Trps confined in the interfacial region connecting Hc-N and Hc-C (Fig. 1).

Table 1
Maximal emission wavelengths and intensities of tryptophan intrinsic florescence (e.g. 295 nm) of Hc, Hc-N and Hc-C at 20 °C

	Hc	Hc-N	Hc-C
λ_{max} (nm)	341	341	344
Fluorescence intensity (a.u.)	303	89	83

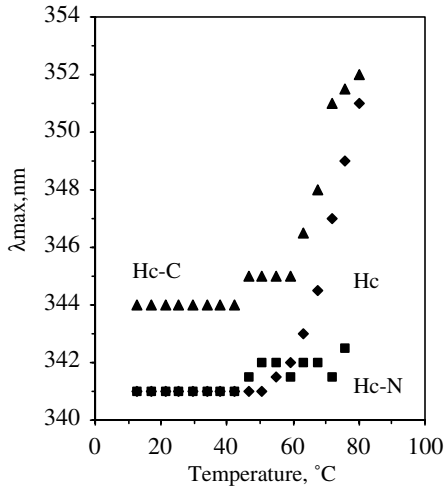

Fig. 4. Thermal denaturation of the proteins. Maximal wavelength emission values of the recombinant proteins Hc (diamonds), Hc-N (squares) and Hc-C (triangle) as a function of temperature.

The fluorescence of Trps is sensitive to their environment. The three proteins had similar maximum wavelength emissions (λ_{max}), although the λ_{max} of Hc-C appeared slightly red-shifted. (Table 1). These values suggest that the Trps share a similar environment within the isolated subdomains or within the whole domain. However, the sum of the fluorescence intensities of both subdomains did not reflect the fluorescence intensity of the entire domain. A partial exposure to the solvent of the Trps of both subdomains could explain this discrepancy. In particular, the exposure of the Trps buried in the hydrophobic interfacial region of the two subdomains (Fig. 1) could explain this loss of fluorescence intensity.

The thermal unfolding of a protein allows to investigate its stability. The results in Fig. 4 show that the λmax of the three proteins remained at the same value between 10 and 50 °C. The three proteins were stable at least 10 °C above 37 °C, the temperature of intraperitoneal injection. Thus, the proteins were accumulated in a thermodynamic state, which most likely corresponded to their respective native states.

Overall, CD and fluorescence data strongly suggest that Hc-N and Hc-C were folded and exhibited the same structural features whether isolated or within Hc. The differences between both subdomains and the whole Hc domain, observed in the near-UV region of the CD spectra and for fluorescence intensities, may be ascribed to the Trps embedded in the apolar interfacial region of Hc, connecting Hc-N and Hc-C. When the subdomains are isolated, the Trps located at the connecting interface (Fig. 1) may enhance their dynamics due to the loss of tight packing and become partially exposed to the solvent,

diminishing both near-UV CD signals and Trp fluorescence intensities, respectively.

3.3. Serum antibody ELISA titers

The immunoglobulin titers of sera from mice vaccinated against Hc, Hc-N and Hc-C were measured by ELISA. Blood samples were taken 17 days after the last vaccination. Total immunoglobulin ELISA titers of mice immunized with Hc-C were slightly but not significantly higher than those of mice inoculated with Hc or Hc-N (Fig. 5A–C). Specific IgG against Hc detected by ELISA were significantly higher in mouse immunized with Hc-C compared to immunizations with Hc and Hc-N, which induced similar IgG levels (Fig. 5D–F). Specific Hc IgM were not significantly detected in any experiment, even at 1/50 serum dilution. Double and triple immunizations provided an ELISA response in total immunoglobulins and IgG significantly higher than the single immunization.

3.4. Protection of mice against challenge with BoNT/A

Single and multiple immunizations have been performed in mice and protection has been monitored by challenge with BoNT/A (10^3 MLD50/mouse) by intraperitoneal injections. A single immunization with Hc induced a protection level, which increased with increasing vaccination doses from 30% with 100 ng/animal to 90% with 5 μg/dose. Full protection was achieved with 10 μg of Hc. The protection levels with the various amounts of the whole Hc domain (Table 2) were similar to those already reported by Byrne and Smith [10]. In single and double immunization experiments, equivalent protein amounts of Hc-N and Hc-C did not provide any significant protection in mice. The highest tested amounts of Hc-N and Hc-C (10 μg) did not give any protection, whereas the same dose of Hc protected 100% of the mice. In a triple immunization experiment with 10 μg of recombinant protein, Hc and Hc-C yielded a 100% protection, whereas the same amount of Hc-N only protected 30% of the mice using a challenge of 10^5 MLD50/animal. These results indicate that the individual subdomains induced a poor protection compared to the whole Hc domain.

Since both Hc-N and Hc-C seemed required to elicit a full protection of the animals, we tested whether the simultaneous injection of the two isolated subdomains induced the same level of protection as the whole Hc. Single immunizations with Hc-N plus Hc-C (2.5 and 5 μg/mouse) did not elicit any protection, whereas equivalent amounts of Hc (5 and 10 μg/mouse) induced 90% and 100% protection, respectively (Table 2).

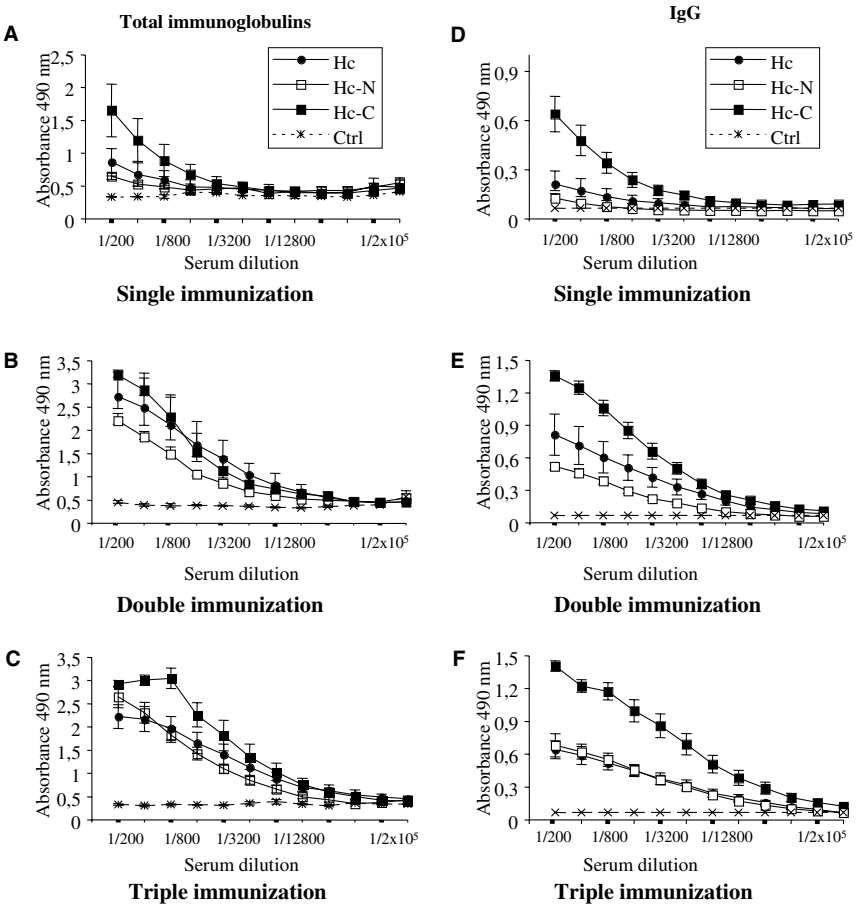

Fig. 5. ELISA titers of sera from mice immunized with Hc, Hc-N and Hc-C. The results are expressed as absorbencies at 490 nm. Serial twofold dilutions of sera starting at 1:200 were used. Mean values and standard errors are shown for 10 mice sera. Ctrl are ELISA titers of sera from non-immunized mice.

Table 2
Efficacy of Hc, Hc-N, and Hc-C vaccines in mice

Antigen	Protein amount (μg)	No. of survivors/Total mice (%)
Single immunization, challenge 20 days later with 10^3 MLD/mouse		
Hc	0.1	6/20 (30%)
	0.3	4/6 (66%)
	1	17/26 (65%)
	5	9/10 (90%)
	10	10/10 (100%)
Hc-N	0.1	0/20
	0.3	0/6
	1	1/26 (3.8%)
	5	0/10
	10	0/10
Hc-C	0.1	0/20
	0.3	0/6
	1	3/26 (11%)
	5	0/10
	10	0/10
Hc-N + Hc-C	2.5 + 2.5	0/10
	5 + 5	0/10
Double immunization with 2 weeks interval and challenge with 10^3 MLD/mouse 20 days later		
Hc	0.3	5/5
	1	5/5
Hc-N	0.3	0/5
	1	0/5
Hc-C	0.3	0/5
	1	0/5
Triple immunization with 2 weeks interval and challenge with 10^5 MLD/mice 20 days later		
Hc	10	10/10
Hc-N	10	3/10
Hc-C	10	10/10

Mice were injected with Hc, Hc-N, Hc-C or a combination of Hc-N and Hc-C in single (A), double (B) or triple (C) immunizations with Alugel, and challenged with BoNT/A as indicated. The numbers of survivors from the total number of challenged mice are recorded.

3.5. Neutralizing antibodies

Like for tetanus, the protection against botulism acquired by vaccination results from neutralizing anti-toxin antibodies in the blood circulation [21]. Therefore, neutralizing antibodies against BoNT/A have been assayed in sera of immunized mice, using the protection test in mice as described in Section 2. Neutralizing antibody titers increased with the number of immunizations and the highest levels were obtained after a triple immunization (Table 3). In agreement with the protection assays against a BoNT/A challenge (Table 2), mice immunized with the whole Hc showed the highest levels of neutralizing antibodies in the serum, whereas immunizations with Hc-N induced the lowest levels (Table 3). Hc-C was more potent than Hc-N to induce neutralizing antibodies. Similar results were obtained in rabbits immunized three times (T_0, $T_0 + 4$ weeks and $T_0 + 6$ weeks) with 100 μg of recombinant protein associated to Freund's adjuvant at each injection. Titers of neutralizing antibodies against BoNT/A were 20 000 in the serum of rabbit immunized with Hc, 200 with Hc-N immunization, and 2000 with Hc-C immunization.

Table 3
Serum neutralizing antibody titers of mice immunized once, twice or three times with 1 μg of Hc, Hc-N or Hc-C. Neutralizing titers, expressed as the inverses of the highest serum dilutions which neutralized 10 MLD of BoNT/A, are the mean of five mice

Antigen	Single immunization	Double immunization	Triple immunization
Hc	20	640	8000
Hc-N	0	20	320
Hc-C	0	160	1280

4. Discussion

We have investigated the possibility to use fragments of the BoNT/A Hc domain to generate recombinant vaccines or antigens for immunizing animals for the production of protective antibodies. Our results show that Hc and its two subdomains, Hc-N and Hc-C can be efficiently produced as His-tag fusion proteins in *E. coli*. CD and fluorescence studies have shown that the three proteins were folded and stable. The proteins elicited serum immune responses in mice as tested by ELISA. The levels of antibodies determined by ELISA increased according to the number of immunizations, but no relevant difference in total immunoglobulins was observed in mice immunized with Hc or the two subdomains. Hc-C seemed to be a better immunogen than Hc and Hc-N to induce an IgG response as detected by ELISA. ELISA method measures antibodies that recognize various epitopes on the antigen molecule but is not predictive of the protection induced against the toxin. Indeed, only the whole Hc domain conferred a high protection in mice against a challenge with BoNT/A, whereas

the two individual subdomains were much less efficient. Hc-C was found to protect mice only when a triple immunization procedure (10 μg/mouse) was applied, whereas the same amount of Hc was fully protective after a single injection. Hc-N did not induce any significant protection. Levels of neutralizing antibodies in sera of immunized mice corroborated the challenge experiments. Mice immunized with Hc showed the highest serum neutralizing titers. Hc-C was more potent than Hc-N and less efficient than the whole Hc to induce neutralizing antibodies. These results are in agreement with previous epitope mapping studies, suggesting that Hc-C contains the major epitopes involved in protection [11,12,22–25]. However, our results show that Hc-C per se was not sufficient, the whole Hc domain was required to induce a high level of protection in mice.

The neutralization of the initial binding of the toxin to cells is the most efficient way of protection. The neuronal receptor of clostridial neurotoxins has not been fully elucidated. It is supposed to correspond to sialo-gangliosides and a protein [26]. The protein receptor has been characterized for BoNT/B and BoNT/G only, which is synaptotagmin I and II, but not for the other BoN/T nor for TeTx [27,28]. In TeTx, which is structurally related to BoNT, it has been reported that Hc-C is necessary and sufficient to bind to the neuronal cell surface and to interact with the dual receptor consisting of polysialoganggliosides and a 15 kDa putative protein receptor, whereas Hc-N does not show any specific binding [29]. Cocrystal structure with synthetic ganglioside showed that TeTx Hc-C contains multiple ganglioside binding sites [30]. TeTx binds simultaneously two ganglioside molecules [31], whereas BoNT/A and BoNT/B only contain one ganglioside binding site [32]. In BoNT/B, the ganglioside binding site was mapped in Hc-C [33]. A similar binding structure is probably conserved in BoNT/A (residues 1266–1272) [30]. For all the clostridial neurotoxins, the binding site(s) to the protein receptor moiety has not been mapped. The multiple interactions between the neurotoxin and the dual receptor, ganglioside and protein, account for the findings that single monoclonal antibodies weakly neutralize BoNT/A, whereas an association of three monoclonal antibodies against Hc is efficiently protective [25,34]. The fact that the whole Hc, but not isolated Hc-N or Hc-C, induced efficient protection may suggest that both subdomains are involved in the receptor binding.

Surprisingly, we found that the co-injection of Hc-N and Hc-C was less efficient than the injection of the whole Hc domain to generate protective antibodies. Thus, the native connection of Hc-N to Hc-C is required to induce high protection in mice. This could suggest that the binding site for the highest affinity receptor involves both subdomains and may be located near the interfacial region of the subdomains. Recently, Atassi and Dolimbek have shown that protective antibody epitopes of BoNT/A H-chain are mainly located in the Hc subdomains, most of them belonging to the interfacial region [35]. In the whole Hc domain, Hc-N and Hc-C are tightly packed together, as shown by the crystal structure [6] (Fig. 1) and by the spectroscopy results presented here (Fig. 3D and Table 1). A high fluorescence intensity and a strong near-UV CD signal showed that Trps are buried in the structure and involved in a highly constrained region in the Hc domain, which is not the case in the isolated subdomains. The separation of the subdomains leading to the exposure to the solvent of the Trps located in the interfacial region may explain the decrease of the fluorescence intensity and of the CD signal. Therefore, the isolated subdomains could loose their native structure and, consequently, their conformational epitopes, in the vicinity of the interfacial region due to the absence of stabilizing tertiary interaction between both subdomains. This could explain the requirement for the whole folded Hc domain to induce a high level of protection in mice as discussed above. Alternatively, all potential discontinuous epitopes overlapping Hc-N and Hc-C are lost when the subdomains are isolated, which may explain the incapacity of isolated subdomains to protect mice. Indeed, it has been shown using monoclonal antibody that discontinuous epitopes are involved in protection [34].

In conclusion, we show that the whole structure of BoNT/A-Hc is necessary to efficiently stimulate a protective immunological response, since single immunization with Hc elicited 90–100% protection level, and equivalent amounts of Hc-N, Hc-C or Hc-N plus Hc-C did not induce any protection. Therefore, no smaller recombinant fragment than Hc can be used as a vaccine candidate or antigen for the production of protective antibodies against BoNT/A. Conformational epitopes from Hc are lost when cleaved in its two subdomains. These epitopes seem critical for the protective immunological response. Since the protective response is essentially due to antibodies, which prevent binding of the toxin to its cellular receptor, the site of interaction with the receptor is likely located in the vicinity of the interfacial region of Hc-N and Hc-C. These data provide additional insights to design efficient recombinant vaccine candidates and potent tools to generate neutralizing polyclonal or monoclonal antibodies against botulinum neurotoxins.

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