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Neurotoxins from *Clostridium botulinum* (serotype A) isolated from the soil of Mendoza (Argentina) differ from the A-Hall archetype and from that causing infant botulism

P. Caballero [a, 1], M. Troncoso [b, 1], S.I. Patterson [b], C. López Gómez [a], R. Fernandez [a], M.A. Sosa [b, *]

[a] Área de Microbiología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina
[b] Laboratorio de Biología y Fisiología Celular, Instituto de Histología y Embriología, FCM, Universidad Nacional de Cuyo, Mendoza, Argentina

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ABSTRACT

The type A of neurotoxin produced by *Clostridium botulinum* is the prevalent serotype in strains of Mendoza. The soil is the main reservoir for *C.botulinum* and is possibly one of the infection sources in infant botulism. In this study, we characterized and compared autochthonous *C. botulinum* strains and their neurotoxins. Bacterial samples were obtained from the soil and from fecal samples collected from children with infant botulism. We first observed differences in the appearance of the colonies between strains from each source and with the A Hall control strain. In addition, purified neurotoxins of both strains were found to be enriched in a band of 300 kDa, whereas the A-Hall strain was mainly made up of a band of ~600 kDa. This finding is in line with the lack of hemagglutinating activity of the neurotoxins under study. Moreover, the proteolytic activity of *C. botulinum* neurotoxins was evaluated against SNARE (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) proteins from rat brain. It was observed that both, SNAP 25 (synaptosomal-associated protein 25) and VAMP 2 (vesicle-associated membrane protein) were cleaved by the neurotoxins isolated from the soil strains, whereas the neurotoxins from infant botulism strains only induced a partial cleavage of VAMP 2. On the other hand, the neurotoxin from the A-Hall strain was able to cleave both proteins, though at a lesser extent. Our data indicate that the *C.botulinum* strain isolated from the soil, and its BoNT, exhibit different properties compared to the strain obtained from infant botulism patients, and from the A-Hall archetype.

1. Introduction

Clostridium botulinum (Cb) is a spore-forming Gram positive bacillus that produces botulinum neurotoxins (BoNT). BoNT are responsible for the fatal neuroparalytic disease botulism. BoNT enter nerve terminals and cause a prolonged neurotransmitter exocytosis blockade, resulting in an impairment of muscle contraction and autonomic nerve functions (reviewed by Zhang et al., 2010). To date, two forms of the disease have been described: the one caused by the preformed toxin, including botulism caused by contaminated food intake, and the toxo-infection which is caused by bacterial colonization of the intestine and the subsequent *in-situ* release of BoNT. The latter category includes the infant botulism (IB), which is one of the most frequent forms of botulism worldwide affecting infants under one year of age (Cox and Hinkle, 2002; Koepke et al., 2007; Fenicia and Anniballi, 2009). Spores of BoNT-producing clostridia are present in the environment and can be found in the dust both domiciliary and peridomiciliary, where the soil appears to be the most important source of contamination (Thompson et al., 1980).

Seven serotypes (A-G) of BoNT have been identified based upon their antigenicity (Arnon et al., 1979). Each serotype is produced by a different strain of *Clostridium botulinum (Cb)*, with all exhibiting a high amino-acid sequence homology (Hill et al., 2007; reviewed by Peng Chen et al., 2012). Four serotypes (A, B, E and rarely F) are known to cause human botulism (reviewed by Hambleton, 1992; Montal, 2010; Peng Chen et al., 2012). In turn, several subtypes have been identified from these serotypes (Smith et al., 2005; Arndt et al., 2006; Carter et al., 2009; Umeda et al., 2009; Jacobson et al.,

* Corresponding author.
E-mail address: msosaescudero@yahoo.com.ar (M.A. Sosa).
[1] They contributed equally to this work.

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2011; Peng Chen et al., 2012). Each BoNT is synthesized as a harmless single polypeptide chain with a molecular mass of ~150 kDa (protoxin). The inactive precursor protein is cleaved by proteases into two active domains, a 50-kDa light chain (LC) and a 100 kDa heavy chain (HC) linked by an interchain disulfide bond (holotoxin). After reduction of the disulfide bond, the fully active toxin is generated. However, to reach their targets (peripheral nerves), toxins need to cross the epithelial barrier of the digestive tract. To this end, BoNTs associate with non-toxic, non-hemagglutinin (NTNH) and some also with hemagglutinin components (NTH). This association gives rise to three complex forms; a protein of 300 kDa (12S) associated with NTNH (toxin M), a protein of 600 kDa (16S) associated with both NTNH and NTH components (toxin L), and a 19S complex, which is thought to be a 16S dimer (toxin LL) (reviewed by Fujinaga, 2010). The *Cb* serotype A, (subtype A1 and A5) produces the three types of complexes (Carter and Peck, 2015), while serotypes B, C and D only produce the 12S and 16S complexes. In turn, the subtype A2-A4, and the serotypes E and F, only produce the 12S complex (Sakaguchi, 1982; Oguma et al., 1999; Poulain et al., 2008; Carter and Peck, 2015).

It is believed that the component NTNH confers proteolytic resistance to BoNTs in the gastrointestinal tract, whereas NTH may play a role in the toxin internalization by the intestine epithelial cells (Fujinaga et al., 1997).

Although the lethal doses of BoNTs in humans are not known, they are assumed to be very similar to those of mice, except for BoNT/D, which is not harmless to humans (Coffield et al., 1997). Arnon et al. (2001) have estimated a LD_{50} of 1 ng/kg in humans.

BoNTs cause flaccid paralysis through an acetylcholine release blockade at the neuromuscular junction in peripheral α-motor neurons. This blockade is accomplished through the cleavage of SNARE proteins (reviewed by Anhert-Hilga et al., 2013) by the LC zinc metalloprotease domain. Thus, serotypes A, C, and E cleave SNAP25, while serotypes B, D, F, and G cleave VAMP-2, and serotype C also cleaves syntaxin 1a (Schiavo et al., 2000; Zhang et al., 2010).

Epidemiological and molecular studies have correlated the prevalence of clostridia producing different botulinum neurotoxin serotypes with geographical regions, and with the presence of disease. Thus, in Argentina the serotype A is prevalent and mostly linked to IB (Lúquez et al., 2005), being the principal route of entry through ingestion of spores from the soil.

In order to identify the soil (a *Cb* reservoir) as the main infection source for IB in the province of Mendoza (Argentina), we characterized and compared *Cb* strains and their respective neurotoxins in bacterial isolates obtained from the soil and those obtained from the fecal samples of IB patients.

2. Materials and methods

2.1. Obtention of native strains of C. botulinum

Ten native strains of *Cb* were isolated, five from fecal samples obtained from infants with botulism (IB*Cb*) and five from soil samples (S*Cb*) of different areas in the province of Mendoza (Argentina) (Bianco et al., 2008; 2009). Strain A-Hall was used for comparison. These strains were maintained lyophilized until used. Strains were resuspended in chopped-meat medium (Giménez and Ciccarelli, 1970) under anaerobic conditions. After 48 h incubation at 35 °C, the purity of strains was assessed on 4% agar plates and egg yolk agar under anaerobic conditions and 1.5% agar (Difco, USA) under aerobic conditions (Dezfulian et al., 1981).

In order to check the mobility of *Cb*, selected strains were subcultured in 1.5% agar for 24–48 h at 35 °C and swarming observation, and subsequently subcultured in 4% agar under anaerobic conditions (48 h at 35 °C) to study the colony morphology (Cato et al., 1986). The colonies formed were photographed with a Nikon COOLPIX 5600 digital camera mounted on a Zeiss Stemi 1000 stereoscopic magnifier.

2.2. Obtention and purification of BoNT from the native strains of Cb

Strains isolated from soil and IB feces were maintained in chopped-meat medium broth with reseeding every 24 h. To obtain the toxin, an aliquot of medium was inoculated into 250 ml of toxin production medium (10 mM Na_2HPO_4, pH 7.2, containing 24% triptycase, 5% proteose peptone, 5% yeast extract, and 5% glucose) and incubated under anaerobic conditions at 35 °C for 96 h. The broth was then cleared by centrifugation at 10,000*g* at 4 °C for 30 min, and the proteins were precipitated with ammonium sulfate (60% saturation) for 24 h at 4 °C with continuous gentle stirring (Sakaguchi, 1982). Crude toxin was then pelleted at 10,000 *g* at 4 °C. Pellets were then dissolved in phosphate buffer (0.03 M, pH 6.8) and then dialyzed for 24 h against 2 mM phosphate buffer (pH 6.8), with three changes.

2.3. Toxicity of BoNTs in mice

The potency of BoNTs was evaluated by intraperitoneal inoculation (ip) of female Swiss-Webster mice weighting 18–22 g each (n = 10), and the LD_{50} was estimated according to Reed and Muench (1938). Animal handling was carried out in accordance with local and national regulations for laboratory animal use. The protocol was approved by ICCULA (Institutional Commission for the Care and Use of Laboratory Animals). Specific activity of the BoNTs was valued as the toxic activity per mg protein.

2.4. Hemagglutination assay

The hemagglutination assay was carried out with the native (from S*Cb* and IB*Cb)* or archetype BoNTs. The assay was performed according to Miyata et al. (2012), with slight modifications. Briefly, 2-fold serial dilutions (starting at 0.5 μg/μl) of either the purified native BoNTs or the archetype BoNT were prepared in phosphate buffered saline (PBS: 0.15 M NaCl, 20 mM NaH_2PO_4, pH 7.0) in 96-well microtitre plates. Then, an equal volume of pre-washed human erythrocytes (O-positive) suspended in PBS (2.5% v/v) was added to each well. After incubation for 2 h at room temperature, the hemagglutination titre was expressed as the reciprocal value of the maximal dilution (2^n) that produced agglutination. All the assays were carried out in duplicate in three independent experiments.

2.5. Electrophoresis

SDS-PAGE was performed under non-reducing conditions according to Laemmli (1970), using 6% polyacrylamide gels. Samples (~25 μg protein) of either the native (S*Cb* and IB*Cb*) or the A-Hall toxins were loaded on the gel and run for 90 min at 25 mV. Protein bands were visualized with Coomassie Brilliant Blue. The molecular mass was determined using a HMW Calibration Kit (Amersham GE Healthcare).

2.6. Proteolytic activity of BoNTs

Rat brain was homogenized (1/5 w/v) in buffer H (50 mM Hepes pH 7.1, containing 5 mM NaCl, 0.1% Tween 20, 10 μM $ZnCl_2$ and 5 mM DTT) with a Teflon pestle homogenizer at 4 °C. Homogenates were centrifuged at 1000*g* for 10 min at 4 °C and the post-nuclear fraction was recovered from the supernatant. For evaluating the

proteolytic activity, purified neurotoxins (2 μg protein) from either IB*Cb*, S*Cb* or A-Hall strains were incubated with aliquots (30 μg protein) of the post-nuclear fraction for 2 h at 25 °C in 20 μl buffer H. The reaction was stopped with loading buffer and the samples were resolved in a 12% SDS-PAGE. After electrophoresis, proteins were electrotransferred onto a 0.2 μm pore diameter nitrocellulose membrane (Amersham Hybond, GE Healthcare), and processed for immunodetection with an anti-SNAP 25 (1:2000) or an anti-VAMP 2 mAb (1:5000) (both from Synaptic Systems, Göttingen, Germany) according to Alberdi et al. (2005), and followed by incubation with an HRP-conjugated anti-mouse IgG (1:5000) (Sigma Chemicals, St. Louis, MO). Specific bands were detected by the enhanced chemiluminescence method (Romano et al., 2005), and quantified by densitometric scanning with the Image Quant LAS 4000 software. Before immunodetection, membranes were stained with Ponceau S as transference control.

2.7. Other procedures

Bacterial morphology from each colony (4% agar) was studied by staining with Gram Nicolle. Protein concentration was determined the Lowry method (Lowry et al., 1951).

2.8. Statistics

Data were analysed by the Tukey-Kramer multiple comparisons test, and the level of significance was set at $P < 0.05$.

3. Results

3.1. Morphological characterization of the isolated colonies

The S*Cb* strain (Fig. 1 A), but not the IB*Cb* strain (not shown), displayed a swarming growth pattern as a thin film on 1.5% agar. When grown on 4.0% agar, S*Cb* strains evinced 2–2.2 mm diameter colonies, with a predominantly irregular shape with high, refringent borders, and with an opaque, and depressed center (Fig. 1B). The IB*Cb* strain formed smaller colonies (1.2–1.5 mm diameter) with refringent, mottled margins, and a mottled and an embossed center (Fig. 1C), similar to the A-Hall archetype (Fig. 1D). Interestingly, the Gram staining revealed that S*Cb* is mostly sporulated, whereas A-Hall and IB*Cb* were predominantly bacilli (Fig. 2).

3.2. Characterization of BoNTs

BoNTs were purified from the culture media following a standard protocol. All BoNTs proved to be toxigenic in mice and neutralized by botulinum antitoxin type A. It is noteworthy that the toxicity of BoNTs purified from S*Cb* and A-Hall was significantly higher than that from IB*Cb* (Table 1). The purified toxins were also analysed by electrophoresis, to find that the BoNTs obtained from IB*Cb* and S*Cb* were enriched in a band of ≈300 kDa, resembling the toxin associated with NTNH component (toxin M) (Fig. 3). However, a band of ~600 kDa, which is evident for the A-Hall toxin, was not observed for IB*Cb* and S*Cb* toxins, indicating absence of NTH component. The lack of the NTH was corroborated by the lack of hemagglutinating capacity of these toxins (Table 1).

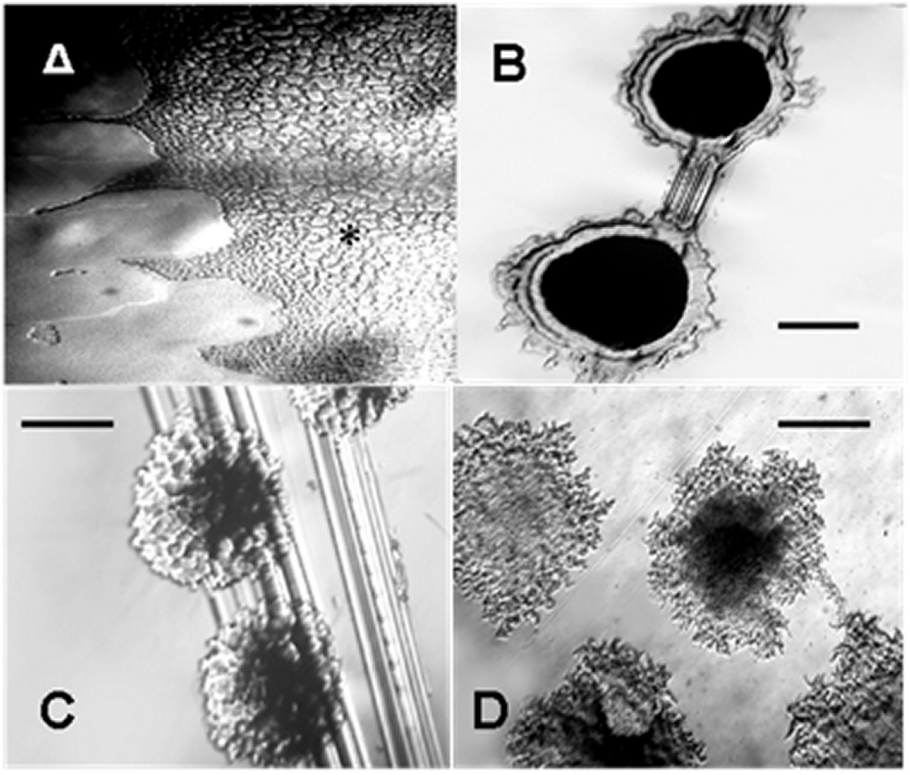

Fig. 1. Morphological features of the *Cb* isolated colonies. (A) S*Cb* strain (asterisk) grown on 1.5% soft agar. (B) S*Cb*, (C) IB*Cb* and (D) A-Hall strains grown on 4% hard agar, as described in Materials and Methods. Scale bars: 1 mm.

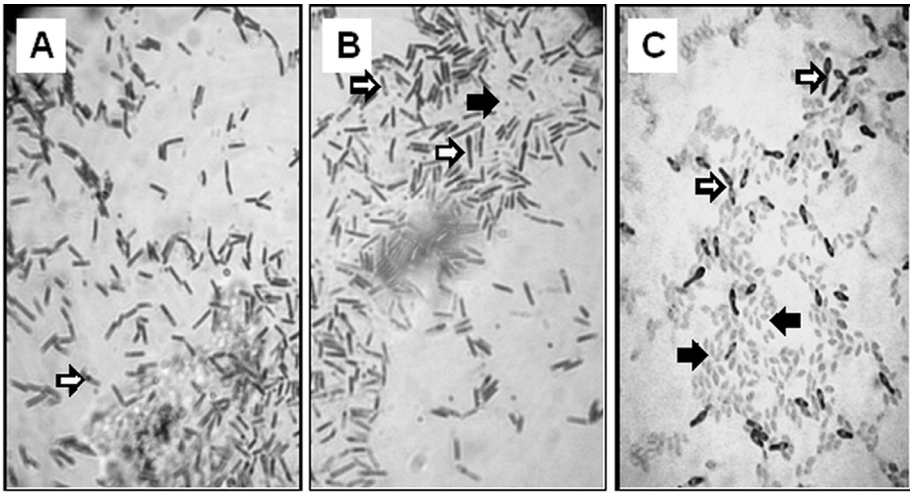

Fig. 2. Bacterial morphology from either IB*Cb* (A), A-Hall *Cb* (B) or S*Cb* (C) colonies. Bacteria were stained with Gram and observed under light microscopy. Magnification: 1000×. White arrows indicate sporulating bacilli and black arrows the spores.

Table 1
Biological parameters of NTBo from different *Cb* strains.

Strain (Mendoza district)	TA (DL_{50}/ml) × 10^6	STA (DL50/mg protein) × 10^6	HA titer
IB*Cb* 82/4 (Guaymallén, 2004)	1942	1739	0
IB*Cb* 84/3 (Tupungato, 2004)	2400	1739	0
IB*Cb* 95/5 (Tupungato, 2005)	3394	2373	0
IB*Cb* 96/3 (Tupungato, 2006)	2673	2138	0
IB*Cb* 99/5 (Tupungato, 2006)	2350	1556	0
S*Cb* 635/3 (San Rafael, 1978)	5800	3333	0
S*Cb* 641/3 (San Rafael, 1978)	6034	3315	0
S*Cb* 1884/2 (Tupungato, 2002)	7010	4249	0
S*Cb* 1891/5 (Tupungato, 2006)	6936	3875	0
S*Cb* 1935/3 (Tupungato, 2008)	7522	5379	0
A-Hall	10,267	4688	2^{11}

TA: toxin activity; STA: specific toxin activity; HA: hemagglutinin activity. IB*Cb:* Infant botulism *Cb*; S*Cb:* Soil *Cb;* A-Hall: standard strain of *Cb*. Six mice per group were used to evaluate TA.

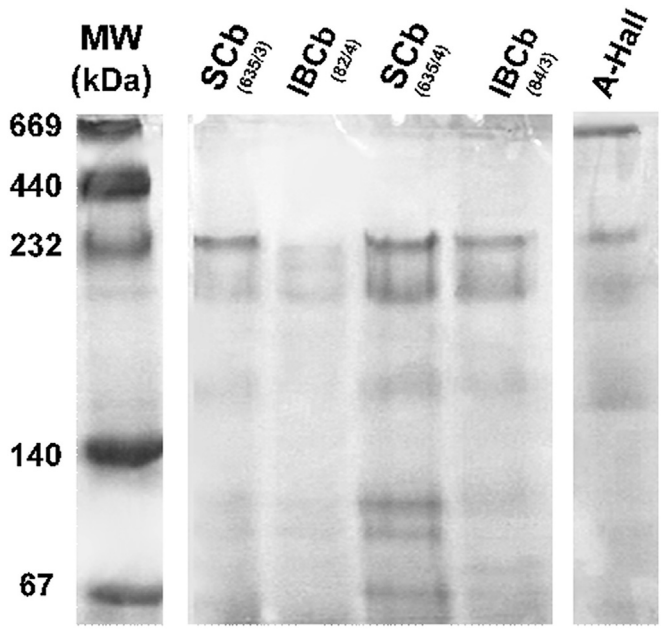

Fig. 3. Electrophoretic analysis of proteins from S*Cb* (635/3 and 645/4 strains), IB*Cb* (82/4 and 84/3 strains) and A-Hall strain. Proteins (25 μg) from each strain were analysed by SDS-PAGE as detailed in Materials and Methods and stained with Coomassie Brilliant Blue.

3.3. BoNTs display a differential activity on SNAREs

To evaluate the proteolytic activity of BoNTs from the *Cb* strains under study, the ability to cleave SNAP-25 or VAMP 2 in rat brain homogenates was assessed, together with immunoblotting and recognition with specific antibodies. Under our experimental conditions, BoNTs from S*Cb* showed a high proteolytic activity on both SNAREs (Fig. 4). The A-Hall BoNT was active on VAMP2, but not on SNAP-25. The IB*Cb* toxin showed an activity pattern that was more similar to the A-Hall strain, being inactive on SNAP-25 and only partially active on VAMP 2 (Fig. 4 B). It is noteworthy that the S*Cb* toxin produced cleavage fragments that were different from those produced by the A-Hall BoNT (Fig. 4 A).

4. Discussion

Infant botulism (IB) has been documented in 26 countries in four continents, with Argentina reporting the second highest number of cases (Fenicia and Anniballi, 2009). Many studies suggest that the main source of contamination are the *Cb* spores resident in the soil, although contaminated honey has been associated with a small number of cases in different countries (Fenicia and Anniballi, 2009). In addition, the presence of BoNT-producing clostridia spores has also been detected in samples of chamomile and linden flowers (Bianco et al., 2008, 2009). In any case, IB occurs when ingested spores germinate, colonize the infant's

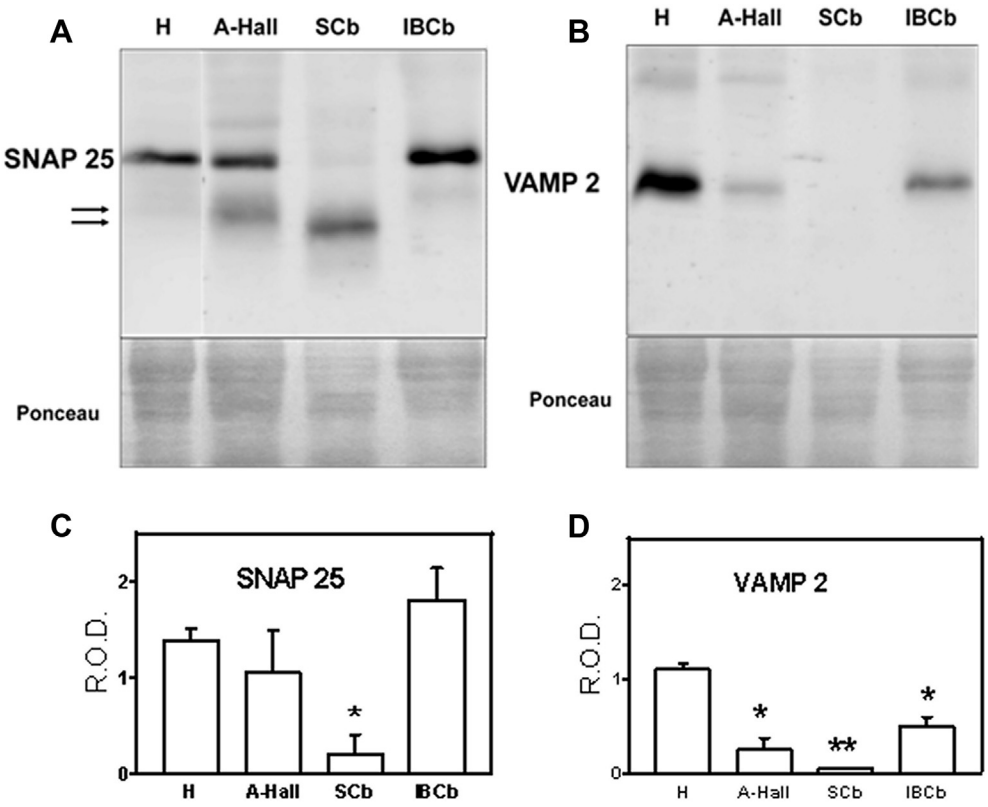

Fig. 4. Proteolytic activity of purified BoNTs. The activity of BoNTs was evaluated on rat brain homogenates as described in Materials and Methods. Homogenates (H) were treated with either SC*b* or IBC*b* toxins, and the results compared with A-Hall BoNT. Proteolytic activity was assayed on either, SNAP25 (A,C) or VAMP2 (B,D), and the protein cleavage was detected by immunoblotting. Protein transference was controlled by staining the membranes with ponceau and of intact proteins were quantified by densitometry. Graphics represent the means ± SD from two independent experiments. R.O.D.: relative optical density. (*) and (**), significantly different with respect to homogenate (H) ($p < 0.05$ and $p < 0.01$ respectively).

gastrointestinal tract, and produce BoNT *in situ*. If the soil is the principal reservoir and source of contamination in IB in Argentina, it would be expected that strains of *Cb* spores in the soil be similar to those isolated from the feces of IB patients. In this study, we found both similarities and differences between strains obtained from the soil and the IB fecal samples. Consistent with an identification of subtype A2, which is prevalent in Argentina (Williamson et al., 2016) the strains under study only produced the 12S complex (Fig. 3) (Sakaguchi, 1982; Oguma et al., 1999; Poulain et al., 2008), which was corroborated by the absence of hemagglutination. However, we cannot discard the presence of subtypes A3 and A4, since their toxins also lack of hemagglutination component (Smith et al., 2007; Jacobson et al., 2008). A sequence of the toxin gene is required to clarified this. Although both BoNTs exhibited toxigenic activity, the SC*b* was significantly more active than the IBC*b* BoNT, suggesting that either both SC*b* and IBC*b* belong to different subtypes or that the IBC*b* is an attenuated version of SC*b* probably arising upon travelling through the digestive tract. The high toxicity of the SC*b* strain could also be related to the high capacity of the BoNT to enter presynaptic neurons, as it occurs with subtype A2 (Pier et al., 2011). The latter difference was also reflected in the proteolytic capacity of the two toxins, since unlike IBC*b* BoNT, SC*b* BoNT was able to cleave SNAP25. The low proteolytic activity of IBC*b* BoNT was not ascribed to an inactivation process taking place during storage, as toxigenicity remains quantitatively unchanged for up to two years (data not shown).

The results obtained herin demonstrate the existence of phenotypic differences between the two autochthonous toxins. These findings are supported by the differences observed in the morphological features of the corresponding colonies. Thus, if we consider the soil as the main reservoir and source of infection of *Cb* for all pathophysiological forms of botulism, we can postulate that the strains suffer subsequent changes during infant intestinal transit.

As mentioned above, SC*b* BoNT was able to cleave SNAP-25, which is consistent with an A serotype (Schiavo et al., 1993). However, this toxin exhibits unprecedented properties, such as the ability to cleave VAMP 2 as well. VAMP 2 is known to be a substrate for other serotypes (Schiavo et al., 1992; Yamasaki et al., 1994). Another surprising feature of SC*b* BoNT is the observed difference in the cleavage product of SNAP 25 compared to the A-Hall archetype. From our observations, it is tempting to speculate that SC*b* is a new subtype belonging to serotype A, with characteristics that have not been described so far. It was reported that subtypes A1-A4 had different cleavage efficiency on SNAP-25 protein (Arndt et al., 2006). Genetic classification of SC*b* and IBC*b* may clarify the difference between the BoNTs.

While some authors have suggested that the NTH component is required for the internalization of the BoNT into cells of the intestinal epithelium, our observations suggest there may be other routes of internalization (Matsumura et al., 2007; Fujinaga, 2010; Lam et al., 2015).

In conclusion, our data indicate that the *Cb* strain isolated from the soil in the province of Mendoza, and its BoNT, exhibit different properties compared to the strain obtained from fecal samples obtained from IB patients, and from the A-Hall archetype. Further molecular and genetics studies are needed to elucidate the identity of both IBC*b* and SC*b*. Finally, a better understanding of *C.botulinum*

and its toxins in every relevant environment could improve our ability to prevent IB.

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