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Type A botulinum neurotoxin complex proteins differentially modulate host response of neuronal cells

Lei Wang [a,*], Yi Sun [a], Weiping Yang [a], Paul Lindo [a], Bal Ram Singh [a,b]

[a] Prime Bio Inc., Dartmouth, MA 02747, USA
[b] Department of Chemistry and Biochemistry, University of Massachusetts Dartmouth, Dartmouth, MA 02747, USA

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ABSTRACT

Type A Botulinum neurotoxin (BoNT/A), the most potent poison known to mankind, is produced by Clostridium botulinum type A as a complex with neurotoxin-associated proteins (NAPs). Currently BoNT/A in purified and complex forms are both available in therapeutic and cosmetic applications to treat neuromuscular disorders. Whereas Xeomin® (incobotulinumtoxin A, Merz Pharmaceuticals, Germany) is free from complexing proteins, Botox® (onabotulinumtoxin A, Allergan, USA) contains NAPs, which by themselves have no known role in the intracellular biochemical process involved in the blockade of neurotransmitter release. Since the fate and possible interactions of NAPs with patient tissues after intramuscular injection are not known, it was the aim of this study to evaluate the binding of BoNT/A and/or the respective NAPs to cells derived from neuronal and non-neuronal human tissues, and to further explore neuronal cell responses to different components of BoNT/A. BoNT/A alone, the complete BoNT/A complex, and the NAPs alone, all bind to neuronal SH-SY5Y cells. The BoNT/A complex and NAPs additionally bind to RMS13 skeletal muscle cells, TIB-152 lymphoblasts, Detroit 551 fibroblasts besides the SH-SY5Y cells. However, no binding to these non-neuronal cells was observed with pure BoNT/A. Although BoNT/A, both in its purified and complex forms, bind to SH-SY5Y, the intracellular responses of the SH-SY5Y cells to these BoNT/A components are not clearly understood. Examination of inflammatory cytokine released from SH-SY5Y cells revealed that BoNT/A did not increase the release of inflammatory cytokines, whereas exposure to NAPs significantly increased release of IL-6, and MCP-1, and exposure to BoNT/A complex significantly increased release of IL-6, MCP-1, IL-8, TNF-α, and RANTES vs. control, suggesting that different components of BoNT/A complex induce significantly differential host response in human neuronal cells. Results suggest that host response to different compositions of BoNT/A based therapeutics may play important role in local and systemic symptoms in patients.

Abbreviations: BoNT, botulinum neurotoxin; BoNT/A, botulinum neurotoxin type A; NAPs, neurotoxin-associated proteins; IL-6, interleukin 6; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-alpha; RANTES, regulated upon activation normal T cell expressed and presumably secreted; BSA, bovine serum albumin.

* Corresponding author. Prime Bio, Inc, 86 Faunce Corner Road, Suite 410, Dartmouth, MA 02747, USA. Tel.: +1 508 858 5018; fax: +1 866 709 1689.

E-mail address: lwang@primebio.net (L. Wang).

1. Introduction

Botulinum neurotoxins (BoNTs), the most potent poison known to mankind (Arnon et al., 2001; Gill, 1982), is genetically and immunologically classified into 7 serotypes A to G (Singh and DasGupta, 1989; Simpson, 2004). And recently, a new strain IBCA10-7060 was identified to produce the eighth serotype BoNT/H from a patient with infant

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botulism (Barash and Arnon, 2013). BoNTs act preferentially on peripheral cholinergic nerve terminals to inhibit acetylcholine release resulting in flaccid muscle paralysis. Despite their lethal properties, BoNTs type A and B are used in medical conditions such as muscle hyperactivity, neuromuscular disorders, various types of pain, and treatment of wrinkles (Rohrich et al., 2003; Salti and Ghersetich, 2008).

Among all the toxin serotypes produced by *Clostridium* botulinum, the duration of type A has the most sustained action in both laboratory animals or in human beings (Keller and Neale, 2001; Foran et al., 2003). The BoNT/A consists of the 150 kDa neurotoxin itself and a set of neurotoxin associated complexing proteins (NAPs), comprising hemagglutinins of 17, 23, 33, 48 kDa and a non-toxin non-hemagglutinin of 138 kDa (Inoue et al., 1996; Sharma et al., 2003). While the NAPs do not play a role in toxin-induced blockade of cholinergic neurotransmission, the presence of NAPs protects BoNTs against proteases of the GI tract during oral poisoning thus enhancing the oral toxicity of the neurotoxin significantly (Sakaguchi, 1982). The currently approved therapeutic applications of BoNTs are by injection into targeted sites, and not via oral intake. Thus the role and effects of these associated proteins need to be further investigated. BoNTs, by their bacterial origin characteristic, are immunogenic. Moreover, the large size of both the complex and the neurotoxin subunit increases the chance of an antitoxin response (Critchfield, 2002). It has been reported that after therapeutic administration of BoNT/A-based drugs, flu-like symptoms have been reported in 1.7–20% of patients injected with BoNT/A and in 5–55% of those injected with BoNT/B (Baizabal-Carvallo et al., 2011) Clinical application of BoNTs has also been reported to induce immuno-resistance response from patients (Benecke, 2012). It is unclear which component of the drug, if any, may accentuate the immune response or inflammatory process.

Since the fate and possible interactions of NAPs with patient tissues after intramuscular injection are not known, it was the aim of this study to evaluate the binding of BoNT/A and/or the respective NAPs to cells derived from neuronal and various non-neuronal human cell lines. BoNT/A and,/or NAPs-induced cytokine release was determined in human neuroblastoma cell line SH-SY5Y, which has been extensively used as a cellular model to investigate intracellular mechanisms of drug actions in human neurons (Xie et al., 2010; Biedler et al., 1978). Our analysis indicates that pure BoNT/A, BoNT/A complex, and NAPs bind dramatically differently to cells developed from human neuronal and non-neuronal tissues, and induce different cytokine release from the neuronal cell line SH-SY5Y, suggesting a significant role of host response upon exposure to different components of BoNT/A.

2. Materials and methods

2.1. Materials

The 150 kDa BoNT/A holotoxin and 500 kDa BoNT/A complex were purchased from Metabiologics Inc. (Madison, WI). NAPs were obtained from dissociated BoNT/A Complex as previous established (Sharma et al., 2003). The NAPs pool was created with DEAE-Sephadex A-50 column at pH 5.5 (20 mM sodium phosphate buffer) followed by a final flow through the SP-Sephadex C-50 column equilibrated with the 20 mM sodium phosphate buffer at pH 7.0. All toxins were produced by a Hall A strain of *C. botulinum*. Toxin activities for the holotoxin and the complex were 2.1×10^7 MLD_{50}/mg and 3.6×10^7 MLD_{50}/mg, respectively, according to the manufacturer. The human neuroblastoma cell line SH-SY5Y, human skeletal muscle cell line RMS13, Jurkat cell line TIB-152, and human skin fibroblast cell line Detroit 551 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Tissue culture media were ATCC-formulated Eagle's Minimum Essential Medium (ATCC), RPMI-1640 medium and Ham's F12 Medium (Sigma–Aldrich, St. Louis, MO) with 10% fetal bovine serum (ATCC). AlexaFluor 488 Protein Labeling kit was purchased from Invitrogen to label bovine serum albumin (BSA), BoNT/A, BoNT/A Complex, and NAPs. Other materials and reagents include: Glass chamber slides (Lab-Tek II chamber slide w/cover, Nalge Nunc International, Naperville, IL). 4% Para-formaldehyde (Sigma–Aldrich, St. Louis, MO). Vecta-Mount permanent mounting medium (Vector Laboratories, Inc. Burlingame, CA). miRNeasy Mini Kit (Qiagen). Bio-Plex Precision Pro™ Human Cytokine Assays (27-plex human group I cytokine plus MIG) (Bio-Rad Laboratories, Hercules, CA).

2.2. Cell culture and treatments

All the human neuronal and non-neuronal cell lines were grown and maintained as recommended by ATCC. The SH-SY5Y cell line was derived from human brain neuroblastoma (Ross et al., 1983). Cells were maintained with 10% FBS in 5% CO_2/humidified air at 37 °C. SH-SY5Y cells grew as a mixture of floating and adherent cells. The base growth medium was 1:1 mixture of ATCC-formulated Eagle's Minimum Essential Medium and F12 Medium. To complete the growth medium fetal bovine serum was added to a final concentration of 10%. The TIB-152 cell line is a mutant of Jurkat (Weiss et al., 1984), and originates from acute T cell leukemia by Schneider (Schneider et al., 1977). The TIB-152 cells are grown in suspension culture and the base medium for this cell line was ATCC-formulated RPMI-1640 Medium. To make the complete growth medium, 10% of fetal bovine serum was added to the base medium. RMS13 cell line was established from cells from the bone marrow of a child with rhabdomyosarcoma (Oliner et al., 1992). The base medium for RMI13 cell line was ATCC-formulated RPMI-1640 Medium. To make the complete growth medium, fetal bovine serum was added to a final concentration of 10%. Human skin fibroblast cell line (Detroit 551) was from normal human skin and had a finite lifespan of about 25 serial passages from the tissue of origin (Sugarman et al., 1985).

SH-SY5Y, RMS13, and Detroit 551 were all adhesion cells. These cells were seeded at a density of 2×10^5 cells/well in 4-chamber glass chamber slides and grew for 2 days before treatment with serum-free media containing 5 nM of BoNT/A, BoNT/A complex, or NAPs proteins. 5 nM of BSA in serum-free media was utilized as control culture. TIB-152 were suspension cells, the following procedure from

McFee was used for handling the cells with revision (McFee et al., 1997): TIB 152 cell pellet was obtained from T75 flasks by centrifugation (2500 rpm for 5 min). The cell density was approximately 2×10^6 cells/ml. Cells were re-suspended in serum-free media with 5 nM of BoNT/A complexing proteins.

2.3. Cell binding by fluorescence microscopy

BoNT/A, BoNT/A complex, and NAPs were labeled with AlexaFluor 488 Protein Labeling kit (Invitrogen) according to the manufacturer's protocol. Labeled proteins were purified from Sephadex G-25 column and eluted with PBS buffer, pH 7.4. All labeled proteins were mixed with 20% glycerol and stored at −80 °C for future use. For adhesion cell lines, neuroblastoma SH-SY5Y cells, skeletal muscle RMS13 cells, and skin fibroblast Detroit 551 cells, cells were seeded in 4-chamber glass chamber slides at a density of 2×10^5 cells/well. Cells were grown to confluence then incubated with serum-free media containing 5 nM of AlexaFluor 488 labeled BoNT/A, BoNT/A complex, or NAPs proteins for 1 h in a 37 °C humidified incubator with 5% CO_2. Medium was removed from chamber slides, and then the cells were washed 3 times with Hank's balanced salt solution (HBSS). Cells were then fixed with 4% para-formaldehyde in PBS for 15 min and were washed again with HBSS three times. The sides of the slides were pulled off and cells were mounted with one drop of VectaMount and covered with large cover slip. Nail polish was used to seal the sides. Slides were stored at 4 °C in foil and observed under fluorescence microscope (Zeiss Axiovert microscope with X-Cite® 120Q excitation light source). For lymphoblast TIB-152 Jurkat cells, the suspension cell line, cells were washed twice with HBSS by centrifugation to remove free dye. Cells were re-suspended in 4% Paraformaldehyde for 10 min at room temperature, and were then observed for labeled protein binding under the fluorescence microscope with a hemocytometer which provided an even monolayer of TIB-152 cells.

2.4. Cytokine multiplex ELISA

SH-SY5Y cells were seeded in 24-well plates with approximately 1×10^7 cells/well. Cells were incubated with serum-free media containing 5 nM of BoNT/A, BoNT/A complex, or NAPs, or for control, 5 nM BSA for 48 h. Supernatants were collected and centrifuged at 13,300 rpm with an Eppendorf MiniSpin Plus microcentrifuge for 10 min at 4 °C to clear the precipitate and stored at −80 °C before being used for quantification of secreted cytokines and chemokines. The BioPlex 200 system was utilized for the analysis of Bio-Rad 27-plex human group I cytokine plus MIG. Concentrations of the following inflammatory cytokines were determined: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin,

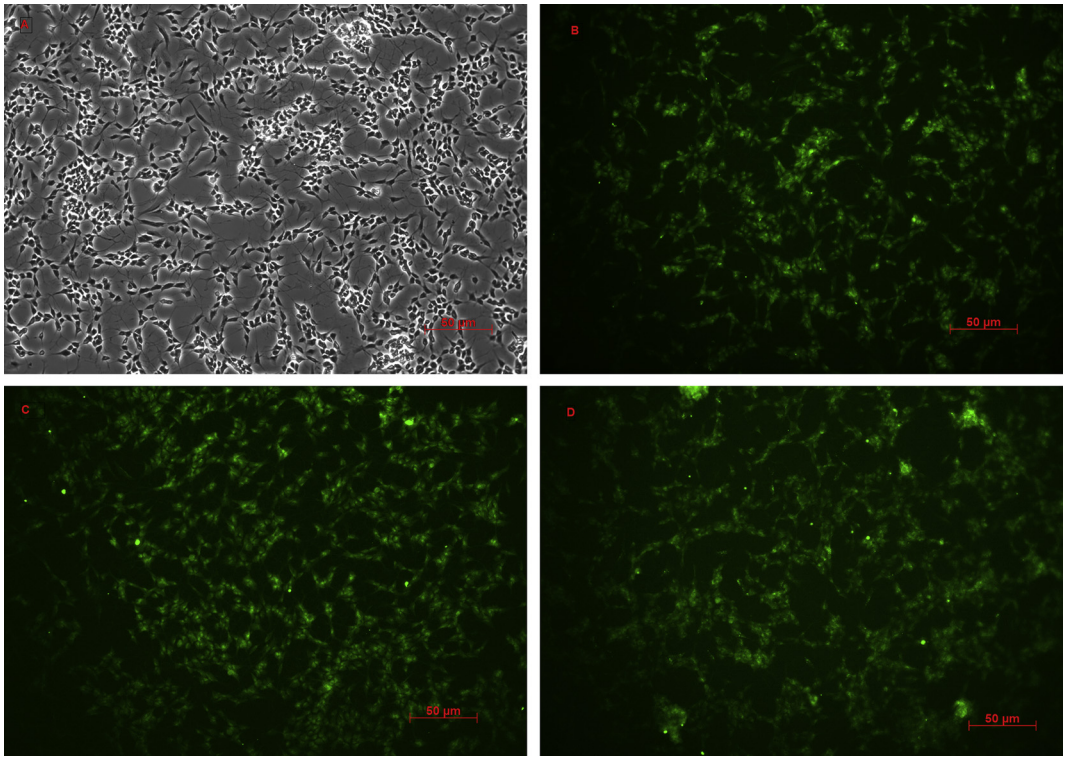

Fig. 1. Fluorescent images showing binding of BoNT/A and its associated proteins to SH-SY5Y human neuroblastoma cells. Bright field (Panel A) and fluorescent images for cells treated with Alexafluor 488 labeled BoNT/A (Panel B), with Alexafluor 488 labeled BoNT/A complex (Panel C), and with Alexafluor 488 labeled NAPs (Panel D). All treatments were performed at 37 °C for 1 h. Fluorescent images were taken using Zeiss Axio fluorescence microscope. Scale bar: 50 µm.

Basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, VEGF, and MIG. The BioPlex assay (Bio-Rad) was performed according to the manufacturer's directions.

3. Results

BoNT/A alone, the complete BoNT/A complex, and the NAPs alone, all bind to SH-SY5Y human neuroblastoma cells (Fig. 1). The complete BoNT/A complex and the NAPs also bind to TIB-152 human lymphoblasts, RMS13 human skeletal muscle cells, and Detroit 551 human fibroblasts, in addition to the neuronal SH-SY5Y cells (Figs. 2–4). Moreover, no binding to these three non-neuronal cell lines was observed with pure BoNT/A (Figs. 2–4). Alexafluor 488 labeled BSA as the control culture did not bind to any of these cell lines (data not shown). Our binding data for pure BoNT/A confirmed previously published research in which the purified BoNT/A bound to cell lines of neuronal origin, but not to those of non-neuronal origin (Kurokawa et al., 1987). But it has not been reported before that in addition binding to human neuronal cells, both BoNT/A complex and NAPs can also bind to non-neuronal cells such as lymphoblasts, skeletal muscle cells, and fibroblasts.

Although BoNT/A in its purified and complex forms all bind to SH-SY5Y, the intracellular responses of the SH-SY5Y cells to these BoNT/A components have not been well studied. Among all the 28 human inflammatory cytokines tested, there were three categories of cytokine release responses: (1) no detectable release, (2) release but no significant differences between BoNT/A, BoNT/A complex or NAPs treatment, and (3) significantly different release induced by BoNT/A, BoNT/A complex or NAPs.

The release of the following thirteen cytokines was below the limit of detection after exposure to different components of BoNT/A associated proteins: IL-1β, MIG, IL-1ra, IL-2, IL-5, IL-17, Eotaxin, basic FGF, G-CSF, GM-CSF, MIP-1α, MIP-1β, and PDFF-BB (Supplementary Table S1). For the following seven cytokines positive releases were detected, but there were no significant changes after the treatment with BoNT/A, BoNT/A complex, or NAPs: IL-4, IL-7, IL-9, IL-10, IL-12, IL-13, and IFN-γ (Table S1).

The cytokines which were significantly induced by different components of BoNT/A and its associated proteins are listed in Table 1. Pure 150 kDa BoNT/A did not significantly increase the release of any inflammatory cytokines from SH-SY5Y cells, compared to BSA control. Exposure to NAPs or BoNT/A complex, however, increased the release of multiple inflammatory cytokines. The release of IL-6, MCP-1, and VEGF were all significantly increased after exposure to BoNT/A complex and NAPs compared with control. In addition, BoNT/A complex induced a significant increase of MCP-1 release compared with NAPs. BoNT/A complex, but not NAPs or BoNT/A, also induced dramatic increase in IP-10, IL-8, TNF-α, and RANTES compared with the control. These results suggest the possibility of NAPs may

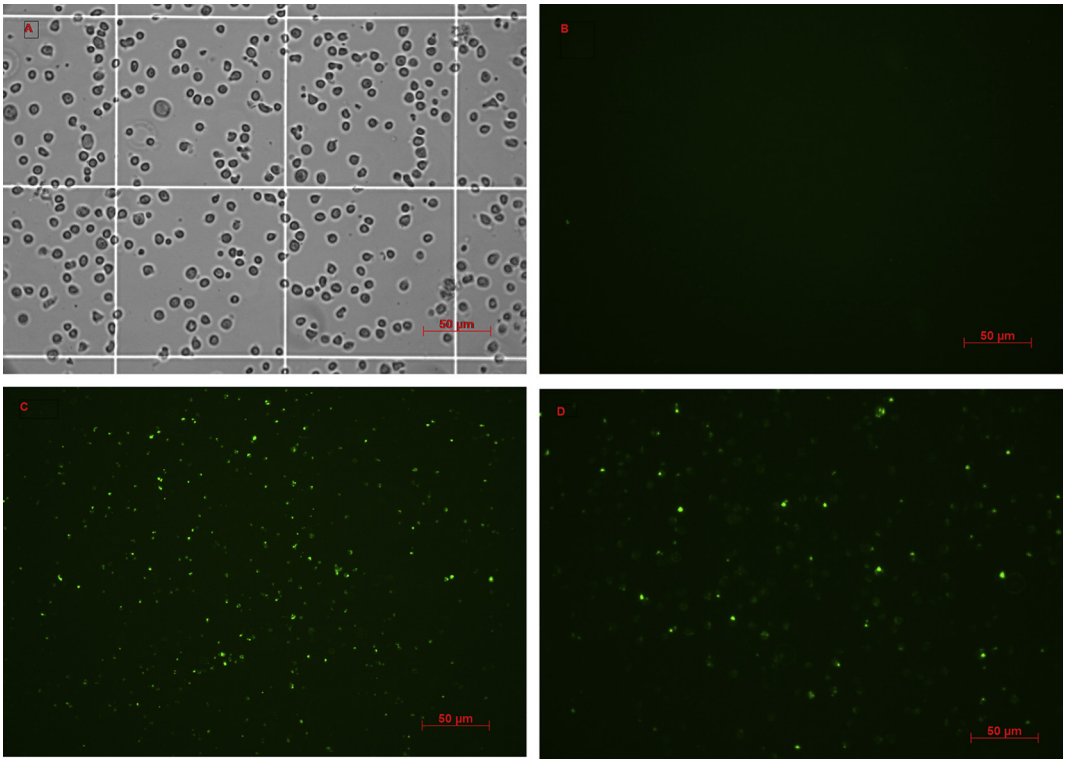

Fig. 2. Fluorescent images showing binding of BoNT/A and its associated proteins to TIB 152 human lymphoblasts cells. Bright field (Panel A) and fluorescent images for cells treated with Alexafluor 488 labeled BoNT/A (Panel B), with Alexafluor 488 labeled BoNT/A complex (Panel C), and with Alexafluor 488 labeled NAPs (Panel D). All treatments were performed at 37 °C for 1 h. Fluorescent images were taken using Zeiss Axiovert fluorescence microscope. Scale bar: 50 µm.

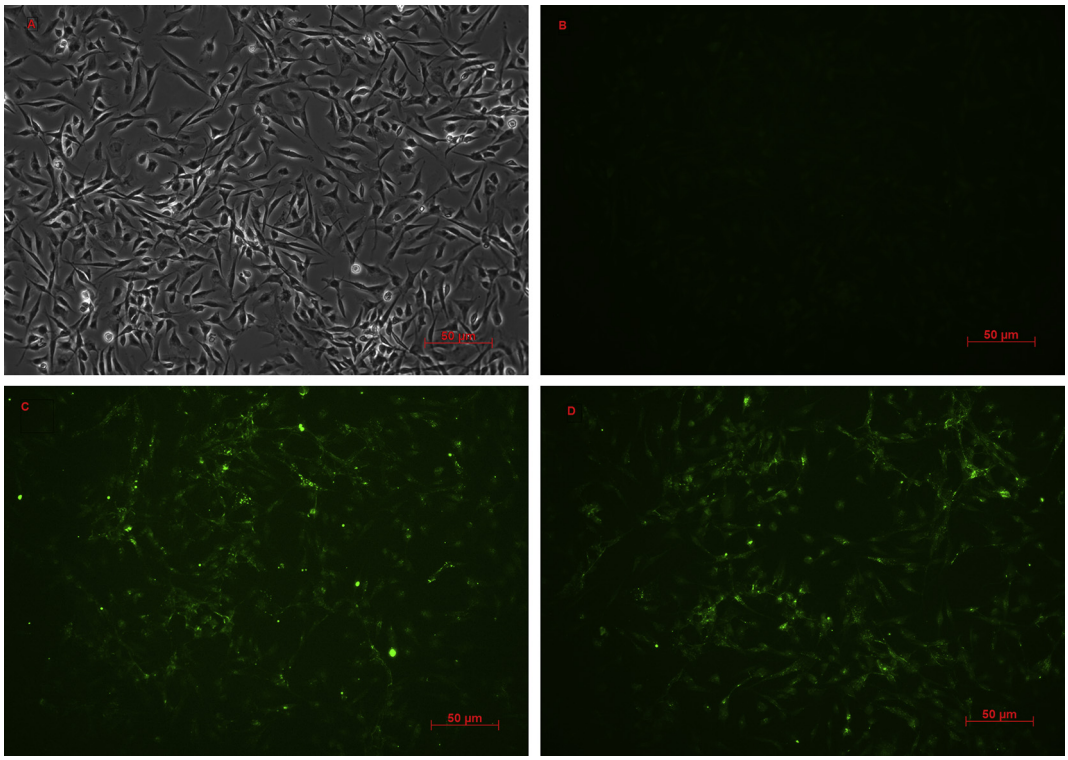

Fig. 3. Fluorescent images showing binding of BoNT/A and its associated proteins to RMS13 human skeletal muscle cells. Bright field (Panel A) and fluorescent images for cells treated with Alexafluor 488 labeled BoNT/A (Panel B), with Alexafluor 488 labeled BoNT/A complex (Panel C), and with Alexafluor 488 labeled NAPs (Panel D). All treatments were performed at 37 °C for 1 h. Fluorescent images were taken using Zeiss Axiovert fluorescence microscope. Scale bar: 50 µm.

contribute to local and systemic inflammatory process after the administration of NAPs-containing BoNT/A drugs in patients.

4. Discussion

Over five million patients are being treated with botulinum neurotoxins globally (Singh et al., 2010), and because of the safety concerns of this being the most toxic substance known to mankind, the United States Food and Drug Administration (US FDA) has designated all botulinum neurotoxin based drugs for black box label (Kuehn, 2009). There have been reports of side effects such as cognition issues and flu-like symptoms from BoNT-based therapeutics (Alam et al., 2002; Costa et al., 2005; Cote et al., 2005), with little knowledge of their causes. Dissecting biochemical effects of each component in active pharmaceutical agent (APA) in BoNT drug products is the first step towards developing a comprehensive understanding of these effects. Since BoNT APA in commercial products contain the BoNT and the NAPs, effects of these two components need to be examined.

A differential binding of BoNT/A complexing proteins to neuronal and nonneuronal cells has not been reported previously. Our data suggest that pure BoNT/A binds specifically to neuronal cells, whereas NAPs bind to neuronal cells as well as, to several non-neuronal cell types. This observation suggests that NAPs may not be just a passive group of associated proteins of BoNT/A complex, rather they at least bind to cells in injected tissues. Previous studies have demonstrated that hemagglutinin (HA) proteins, which are important components in the BoNT/A complex, are important for carbohydrate recognition and can bind to oligosaccharides on erythrocytes through HA-33 (Arndt et al., 2005; Fujinaga et al., 2000; Inoue et al., 2001). A similar mechanism is likely to be involved, although a report had implicated HA-33 binding to one of the known receptors of BoNT/A (Zhou et al., 2005). The signs and symptoms of flu symptoms are ordinarily associated with influenza virus infection (Puzelli et al., 2009). Previous research has shown that HA influences the infectivity of type A influenza virus in dendritic cells (DC). The DC cells play a key role in early phases of the immune response, and subsequently as antigen-presenting cells that activate the adaptive immune response (Hargadon et al., 2011). In addition, our previous study demonstrated that NAPs have stronger immunogenicity over that of purified neurotoxin, thus having a higher potential of BoNT/AC and its associated proteins to induce host immune response (Kukreja et al., 2009). BoNT/A itself appears to be directed to a given cell type through a specific set of gangliosides and specific protein receptors. For example, recent research reports have suggested that the same receptors on neuronal and intestinal cells could drive distinct trafficking pathways for BoNT (Humeau et al., 2000).

A relevant question is what the implications of the binding of BoNT or NAPs to a given type of cells are? BoNT/A binding results in internalization and translocation into the

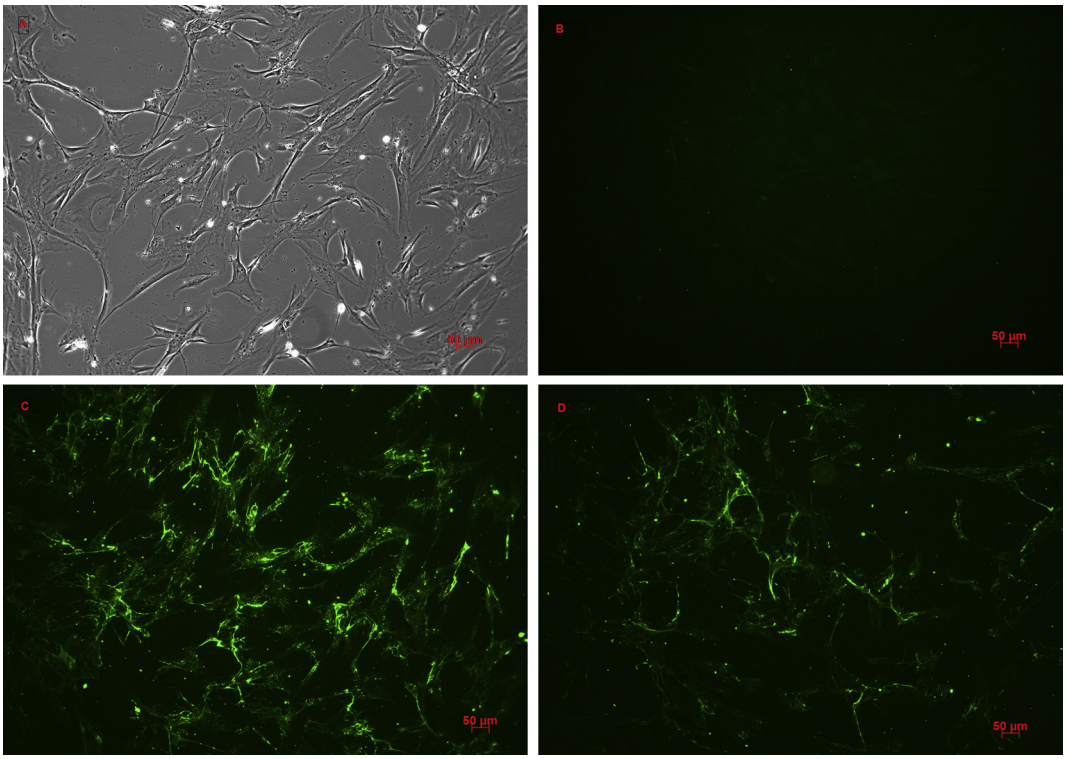

Fig. 4. Fluorescent images showing binding of BoNT/A and its associated proteins to Detroit 551 human fibroblasts cells. Bright field (Panel A) and fluorescent images for treated with Alexafluor 488 labeled BoNT/A (Panel B), with Alexafluor 488 labeled BoNT/A complex (Panel C), and with Alexafluor 488 labeled NAPs (Panel D). All treatments were performed at 37 °C for 1 h. Fluorescent images were taken using Zeiss Axiovert fluorescence microscope. Scale bar: 50 μm.

cytosol where it cleaves SNAP-25 leading to blockage of neurotransmitter release (Sharma et al., 2006; Poulain et al., 2009). We were interested in what other biochemical or physiological response caused by the presence of toxin inside the neuronal cells. Previously we had tested effect on BoNT/A on apoptosis of neuronal cells (Kumar et al., 2012). In this work, we examined cytokine response, and concluded that pure BoNT/A caused virtually no cytokine response after 48 h of incubation (Table 1). However, BoNT/A complex and NAPs exerted substantial yet selective cytokine response, suggesting that NAPs binding, and possible entry into the cell has significant host response. It is, however, notable that NAPs by themselves do not exert as much influence as the BoNT/A complex, except for IL-6 which showed equal response (Table 1). MCP-1 and VEGF were two other cytokines which were induced by NAPs alone, albeit not as strongly as BoNT/A complex. BoNT/A complex and NAPs both contain associated proteins for BoNT/A. However, exposition to BoNT/A complex, but not to NAPs, resulted in significant increase of IP-10, IL-8, IL-15, TNF-α, and RANTES. For the current research, cytokine release was examined after 48 h of incubation. The kinetics of cytokine release have been studied for 24 h to up to one week in lymphocyte (Arva and Andersson, 1999) and kinetics of TNF, IL-6, and IL-8 gene expression after inflammatory stimuli have been shown to have multiple peak at 2–4 h and 24 h (DeForge and Remick, 1991). Although no cytokine release was induced by pure BoNT/A in the current experimental setting, further investigation with different incubation time on complex patterns of cytokine gene expression and production with pure BoNT/A as well as other components of BoNT/A complex is needed.

Higher effect of BoNT/A complex could arise from one or more of the following reasons. One, there is higher level of binding of the BoNT/A in the BoNT/A complex allowing more NAPs to enter the cell. Two, interaction between BoNT/A and NAPs introduce conformational changes which are more critical for triggering cytokine response. Three, there is a physiological link between the effects of BoNT/A and NAPs intracellularly, leading to synergistic host cell response.

A previous study on the co-culture of microglia and SH-SY5Y cells has shown the expression of IL-6, IL-8, and MCP-1 with borrelia burgdorferi stimulation, a spirochete that causes lyme disease, and it is known to potently induce the production of inflammatory mediators in a variety of cells (Myers et al., 2009). Release of MCP-1 from SH-SY5Y has also been reported during the neuroinflammation process (Mitchell et al., 2009).

Physiological role of cytokine release in neuronal cells can be manifold. The presence and activity of pro-inflammatory cytokines IL-1β and TNF-α were first reported in human and rat brain a decade ago (Breder et al., 1988; Plata-Salaman et al., 1988). Cytokine release studies enable us to identify cytokines that are produced specifically upon BoNT/A, its complex, or NAPs stimulation. The SH-SY5Y cell line has been proven to be a useful in vitro

Table 1
Cytokine release analysis of SH-SY5Y cells after 48-h treatment with BoNT/A, BoNT/A complex, and NAPs.

Treatment	IP-10	IL-6	IL-8	IL-15	MCP-1	TNF-α	RANTES	VEGF
Control (BSA)	ND	4.24 ± 1.01	129.2 ± 6.31	ND	1096.25 ± 72.4	27.46 ± 9.91	172.13 ± 5.17	15234.84 ± 831.69
BoNT/A	ND	6.99 ± 1.21	135.35 ± 13.02	ND	1105.07 ± 42.98	10.33 ± 4.79	179.82 ± 6.17	17633.35 ± 1757.27
BoNT/A complex	3355.94 ± 149.88*	13.57 ± 3.79*	1106.66 ± 78.35*	10.6 ± 1.77*	2943.07 ± 166.67*	265.04 ± 11.05*	1108.42 ± 33.99*	25786.35 ± 2556.7*
NAPs	ND	14.05 ± 5.28*	127.93 ± 16.93	ND	1783.18 ± 8.42*	12.41 ± 8.9	184.74 ± 8.79	19679.43 ± 3294.71*

Pure BoNT/A didn't increase the release of most of tested pro-inflammatory cytokines. BoNT/A complex and NAPs both significantly increased the release of IL-6, MCP-1, and VEGF. In addition, BoNT/A complex, but not NAPs, significantly increased the release of IP-10, IL-8, IL-15, TNF-α, and RANTES. (ND: non-detectable signal. Cytokine concentration: pg/ml. Means ± Std Err. *$p < 0.05$ vs. Control BSA, unpaired t-test, $n = 4$).

model for TNF production from neurons and the regulation of that production by alpha2-adrenergic receptor activation (Renauld and Spengler, 2002). Additionally, TNF-α has been shown not only play the critical roles in pathological development and inflammatory induction, but on modulating cell proliferation of neural progenitors in CNS inflammation (Downen et al., 1999; Wu et al., 2000). A TNF-α increase of more than 20-fold in BoNT/A complex treated SH-SY5Y cells compared to BoNT/A or NAPs indicates that BoNT/A complex triggered the release of the cytokine and might account for the proinflammatory response of BoNT/A. Neuron-derived TNF-α may maintain the activity of neurons by sustaining physiologic levels of neurotransmitter release through regulation of adrenergic autoreceptor activity (Ignatowski et al., 1997). Flu like symptoms have been reported in between 1.7 and 20% of patients treated with various preparations of BoNT/A (Baizabal-Carvallo et al., 2011). It has been reported that neurons and glial cells produce cytokines in cell culture, in particular after addition of inflammatory stimuli (Schobitz et al., 1994). A recent published study evaluated blood cytokines of patients following treatment with BoNT and discovered that inflammatory cytokines are increased in many patients following BoNT injection (Baizabal-Carvallo et al., 2013), although clear role of NAPs was not deciphered.

Our study suggested that the observed flu-like symptoms after BoNT/A application may also be the result of inflammation resulting from the inflammatory mediators released in response to some components in the BoNT/A complexing proteins. Due to the fact that in the current study, we utilized laboratory grade pure BoNT/A, BoNT/A Complex, and NAPs instead of commercially available products, and the in vitro concentrations of BoNT/A and associated protein in the current study are higher than the therapeutical use, further research needs to be done for this aspect. For the comparison of current commercially available BoNT/A products, the primary challenge is that the lack of standardized measurement. The units of different BoNT/A products are not interchangeable due to the differences of LD_{50} protocols in house (Sesardic, 2010). We plan to compare the host response of cells to different currently available products and will need to determine a relative equipotency point for these agents during the treatment. It is very important to utilize the concentration in the range of nM in cellular model to avoid false negative results. We will also include fM to pM concentration range, which is therapeutic dose, to facilitate our understanding of clinical observations.

It is concluded that BoNT/A in the complex form with the presence of NAPs protein induced significant inflammatory cytokine release. The presence of NAPs has also been shown to accentuate the immune response to the BoNT/A injections (Siatkowski et al., 1993; Goschel et al., 1997). As the presence of associated proteins within the therapeutic formulation of BoNT/A complex increases the protein load, and may accentuate the immune response or inflammatory process, further experiments to investigate effects of BoNT/A components are warranted. It may help to clarify different physical effects caused by BoNT/A in its purified or complex forms. Clearly understanding the intracellular signaling pathways and the mechanism of the

inflammatory effects of BoNT/A and its associated proteins will reveal the crucial role for each components in both physiologic as well as in pathologic conditions of neurons.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2014.02.004.

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