Development of a delivery vehicle for intracellular transport of botulinum neurotoxin antagonists[1]

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Abstract A targeted delivery vehicle (DV) was developed for intracellular transport of emerging botulinum neurotoxin (BoNT) antagonists. The DV consisted of the isolated heavy chain (HC) of BoNT/A coupled to a 10-kDa amino dextran via the heterobifunctional linker 3-(2-pyridylthio)-propionyl hydrazide. The HC served to target BoNT-sensitive cells and promote internalization of the complex, while the dextran served as a platform to deliver model therapeutic molecules to the targeted cells. To determine the ability of this chimeric glycoprotein to enter neurons, dextran and HC were labeled independently with the fluorescent dyes Oregon green 488 and Cy3, respectively. Internalization of DV was monitored in primary cortical cells using laser confocal microscopy. Incubation of cells for 24 h with DV resulted in discrete punctate labeling of both soma and processes. The Cy3 and Oregon green 488 signals were generally co-localized, suggesting that the complex remained in the same intracellular compartment during the initial 24 h. The DV-associated fluorescence was reduced progressively by co-application of increasing concentrations of unlabeled BoNT/A holotoxin. The results suggest that the BoNT/A HC is able to mediate internalization of a coupled dextran, even though the latter bears no resemblance to the BoNT/A light chain (LC). The HC of BoNT/A thus offers promise as a selective carrier to deliver BoNT antagonists to the nerve terminal cytoplasm for inhibiting the proteolytic activity of internalized BoNT/A LC. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Botulinum neurotoxin; Targeted drug delivery; Laser confocal microscopy; Fluorescent probe

1. Introduction

The botulinum neurotoxins (BoNTs) comprise a family of seven distinct neurotoxic proteins (A–G) that are the most lethal substances known to mankind [1,2]. Exposure to BoNT results in inhibition of acetylcholine release leading to muscle weakness, paralysis, respiratory arrest and death [3]. The toxins are dichain proteins consisting of a ~100-kDa heavy chain (HC) and a ~50-kDa light chain (LC), coupled by a single disulfide bond and non-covalent interactions [4]. The HC is responsible for cell surface binding and internalization and is devoid of toxicity when isolated from the LC [5]. The LC is a zinc metalloprotease that cleaves one of three intracellular proteins responsible for neurotransmitter release: synaptobrevin, syntaxin or synaptosomal-associated protein of 25 kDa [6]. Isolated LC also lacks toxicity due to its inability to gain access to the neuronal cytosol but manifests this property if transported intracellularly by microinjection or liposome delivery [7].

The BoNTs are best known for their role in mediating food-borne, wound and infant botulism [1]. Their high potency and ease of production also render the BoNTs potential biological warfare agents [8]. Although an effective vaccine is available, the relatively low incidence of botulism makes widespread vaccination impractical [9]. In addition, vaccinated individuals would be precluded from the therapeutic benefit of BoNT for treatment of dystonias and movement disorders [10].

Current treatment for BoNT intoxication consists of infusion of trivalent equine antitoxin to neutralize circulating toxin and supportive care [2]. The former approach is limited by a brief therapeutic window, since much of the toxin is internalized by the time symptoms appear [11]. A post-exposure pharmacological treatment for botulism has been pursued for over a decade but has met with only limited success. Difficulties with current drug candidates include low efficacy [12], high systemic toxicity [13,14] and poor bioavailability [15]. Consequently, there is a compelling need for the development of effective BoNT inhibitors and for devising strategies to direct inhibitors into the nerve terminal cytosol. The problems of low efficacy are being addressed by new synthetic approaches [16,17], but specific targeting of drugs will be needed

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[1] The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense.
In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996.

Abbreviations: DV, delivery vehicle; BoNT, botulinum neurotoxin; HC, heavy chain; LC, light chain; PBS, phosphate-buffered saline; PDPH, 3-(2-pyridylthio)-propionyl hydrazide; SEC, size-exclusion chromatography; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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for selective intracellular delivery of sufficiently high concentrations to inhibit the proteolytic activity of the LC.

The current study describes the design of a delivery vehicle (DV) for the selective intracellular targeting of BoNT antagonists. The DV consists of a chimeric glycoprotein in which a 10-kDa amino dextran is covalently attached through a bifunctional linker to the BoNT/A HC. The HC serves to target cells containing BoNT/A surface receptors and to promote internalization of the complex; the dextran serves as an inert carrier of BoNT/A antagonists. To visualize cellular uptake, DV was differentially labeled with the fluorescent dyes Cy3 (Amersham Biosciences, Piscataway, NJ, USA) and Oregon green 488 (2′,7′-difluorofluorescein, Molecular Probes, Eugene, OR, USA), and evaluated in cultured cortical cells. The results indicate that the DV was able to undergo neuronal uptake and that this process was inhibited by the presence of unlabeled BoNT/A holotoxin in a concentration-dependent fashion. The success of the DV prototype suggests that the approach of using a targeted delivery system for botulinum antagonists is feasible.

2. Materials and methods

2.1. Strategy for DV design

A schematic representation of the DV used in the present study is illustrated in Fig. 1. The following steps were involved in the synthesis of this chimeric glycoprotein: (1) labeling of pure BoNT/A holotoxin with the fluorescent dye Cy3, (2) isolation and purification of the Cy3-labeled HC, (3) oxidation of the hydroxyl groups in the dextran to allow attachment of the linker 3-(2-pyridylthio)-propionyl hydrazide (PDPH, Pierce Biochemical, Rockford, IL, USA), (4) labeling of the dextran with Oregon green 488, and (5) coupling of the labeled dextran and HC. Coupling required disulfide exchange of one of four sulfhydryls of the HC with the functional group of the linker to constitute the complete DV. The detailed synthetic procedures are provided below.

2.2. BoNT/A production and purification

BoNT/A was obtained from the Hall strain of *Clostridium botulinum*. Cultures were grown statically in 10-l volumes of toxin production medium consisting of 2.0% NZ TT casein hydrolysate (Sheffield Laboratories, Norwich, NY, USA), 1.0% yeast extract (Difco Laboratories, Detroit, MI, USA) and 0.5% dextrose, pH 7.3–7.4. The cultures were incubated for 4 days at 37°C. BoNT/A was isolated and purified according to the methods of Goodnough and Johnson [18].

2.3. Cy3 labeling of BoNT/A and chain separation

Fluorescent labeling of isolated HC was not attempted since preliminary experiments indicated that this approach resulted in extensive degradation of the HC. Instead, labeling with Cy3 was performed prior to chain separation. Pure BoNT/A in 20 mM sodium borate, 40 mM sodium phosphate (pH 8.4) was labeled with the monoreactive succinimidyl ester of the fluorescent cyanine dye Cy3 using 25 μg dye per mg neurotoxin. The reaction of BoNT/A and Cy3 was carried out overnight at room temperature in the dark. Cy3 reacted with free amines in the toxin and was predominantly associated with the ε-amino groups of lysine residues. Unreacted dye was removed by overnight dialysis at 4°C against 10 vol of 20 mM sodium borate and 40 mM sodium phosphate (pH 8.4) with three buffer changes. Addition of Cy3 resulted in a small reduction in specific toxicity of BoNT/A from 1.05×10^8 to 7.5×10^7 mouse i.p. LD_{50}/mg.

BoNT/A was separated into HC and LC according to the method of Sathyamoorthy and DasGupta [4]. HC purification was determined to be >99% complete by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 2). Purified Cy3-labeled HC was stored as an ammonium sulfate precipitate (39 g solid ammonium sulfate per 100 ml) at 4°C until required for coupling with dextran. The specific toxicity of the purified HC preparations was ≤500 mouse i.p. LD_{50}/mg protein due to contamination by trace quantities of residual holotoxin.

2.4. Dextran preparation

Amine-reactive dextran (Molecular Probes, Eugene, OR, USA) was prepared for conjugation with BoNT/A HC by reaction with sodium periodate followed by addition of the heterobifunctional linker PDPH. Dextran (10 kDa, 15 mg/ml) was first modified by oxidation of hydroxyls using sodium periodate (10 mM final concentration in 100 mM sodium acetate, pH 5.5, for 20 min at 22°C in the dark). Oxidation of vicinal hydroxyl groups to the corresponding aldehydes is required for reaction with the hydrazide portion of the linker. Excess periodate was removed by size-exclusion chromatography (SEC) on Sephadex G25 columns (1 cm×10 cm) equilibrated with 100 mM sodium acetate, pH 5.5. Immediately upon elution, the oxidized dextran was reacted with 5 mM PDPH and maintained overnight at 22°C in the dark. Unreacted PDPH was removed by SEC in a phosphate-buffered saline (PBS) consisting of 20 mM sodium phosphate (pH 7.4) and 150 mM NaCl. The concentration of incorporated PDPH was determined by reduction of eluted dextran with 5 mM dithiothreitol and measuring the increase in absorbance at 343 nm. Modified dextran (13 mg) was reacted with the succinimidyl ester of Oregon green 488 (5 mg) (Molecular Probes) in 3.0 ml of PBS at 22°C overnight in the dark. Unreacted Oregon green 488 was removed by SEC on Sephadex G25 columns equilibrated with PBS plus 1 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.4.

2.5. Conjugation of HC and dextran

Purified HC (ammonium sulfate precipitate) was collected by centrifugation (10 000×*g*, 15 min at 4°C) and dissolved in ice-cold PBS, 2 M urea and 10 mM dithiothreitol. Dithiothreitol and urea were removed from HC preparations by SEC on columns equilibrated with PBS, plus 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO, USA). Fractions containing HC were immediately reacted with labeled dextran. Conjugation was monitored by measuring increases in absorbance at 343 nm. This procedure entails formation of a disulfide linkage between a sulfhydryl group on one of the four reactive cysteine residues of the HC and the sulfur of the PDPH linker associated with the dextran. Reactions were stopped at various time points by addition of methionine to a final concentration of 2 mM to determine optimal coupling times.

2.6. Stoichiometry of dye and linker incorporation

The degree of Cy3 incorporation into HC was estimated spectrophotometrically using an extinction coefficient of 1.5×10^5 M^{-1} cm^{-1} at 552 nm for the dye and 1.4×10^5 M^{-1} cm^{-1} at 278 nm for the HC. A correction of 8% for absorbance of Cy3 at 278 nm was used when determining HC concentration. Fluorescent label density on dextrans was calculated using 8.4×10^4 M^{-1} cm^{-1} as the extinction coefficient of the Oregon green 488 at 494 nm; PDPH linker density was determined using an extinction coefficient of 8.08×10^3 M^{-1} cm^{-1} at 343 nm following reduction with 5 mM dithiothreitol.

2.7. Gel electrophoresis

Protein samples were separated electrophoretically using the Pharmacia Phastsystem (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Precast 10–15% acrylamide gels were stained with 0.1% Coomassie brilliant blue R250 in 16.7% acetic acid/41.7% methanol. Gels were destained in 7.5% acetic acid/25% methanol. Samples for electrophoresis were solubilized in 50 mM Tris–HCl, 5 M urea, 5% SDS, 20% glycerol, pH 6.8. Where indicated, samples were reduced by addition of dithiothreitol at a final concentration of 0.5%. All samples for SDS–PAGE were boiled for 5 min prior to electrophoresis.

2.8. Cell culture

Cultures of dissociated mouse cortical cells were prepared as described by Matthews et al. [19]. Primary mouse cortical cells were obtained from CD-1 mice on embryonic day 18. Tissues were isolated and placed at 37°C in a dissociation medium consisting of Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution containing 1 mM sodium pyruvate, 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-2-ethanesulfonic acid (pH 7.3) and 0.025% trypsin. After 10 min, trypsin was diluted by addition of nine volumes of dissociation medium. Cells were triturated and passed through a 30-μm mesh to remove large aggregates. The suspension was centrifuged at 1500×*g* for 2 min, and the pellet was resuspended in growth medium (Neurobasal medium, 2% B27, 0.5 mM L-alanyl-L-glutamine, 100 U/ml penicillin, 100 μg/ml strepto-

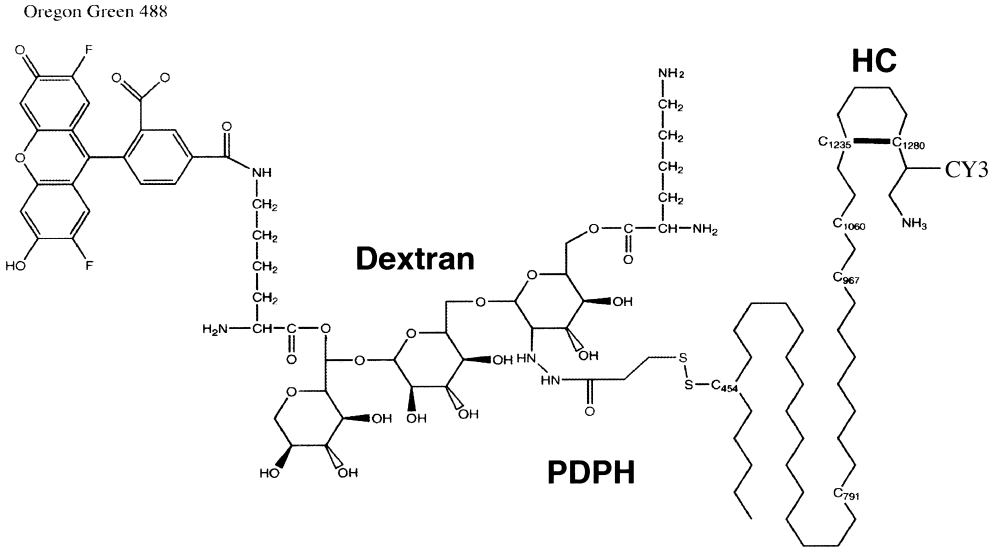

Fig. 1. Schematic representation of the DV for transport of BoNT/A antagonists. The PDPH linker is bound to one of four possible cysteine (C) sulfhydryl groups on the BoNT/A HC. It is shown attached to C454, which normally participates in the disulfide linkage with the LC. Cy3 and Oregon green 488 are bound to ε-amino groups of lysine in the HC and dextran, respectively. The dextran is conjugated to the HC by a C–N bond in one of the glucose residues. On average, each mol of HC contained 2.8 mol of Cy3; each mol of dextran contained 2.3 mol of Oregon green 488 and 3.2 mol of PDPH.

mycin, 0.25 μg/ml amphotericin; Life Technologies) with 1% horse serum. Cells were plated on poly-D-lysine-coated Thermanox plastic coverslips (Nunc, Naperville, IL, USA) in 24-well plates at a density of 10^5 cells/cm^2. Cells were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2/10% O_2/85% air and used 7–9 days after plating.

2.9. Uptake of DV by cortical cells and fluorescent imaging

Cells were exposed to DV, Cy3-labeled HC, or Oregon green 488-labeled dextran in growth medium for 24 h at a concentration of 200 nM at 37°C. Cells were subsequently washed three times with control growth medium and fixed overnight with 2% paraformaldehyde. Plastic coverslips containing fixed cells were mounted between a glass slide and glass coverslip and viewed on a Zeiss LSM laser confocal microscope. Oregon green 488 was excited at 488 nm and observed through a 515-nm cutoff filter. Cy3 was excited at 543 nm and detected with a 565-nm cutoff filter. To minimize photobleaching, Slowfade Light (Molecular Probes) 1:50 was added to the mounting medium. Images were collected with Zeiss LSM software.

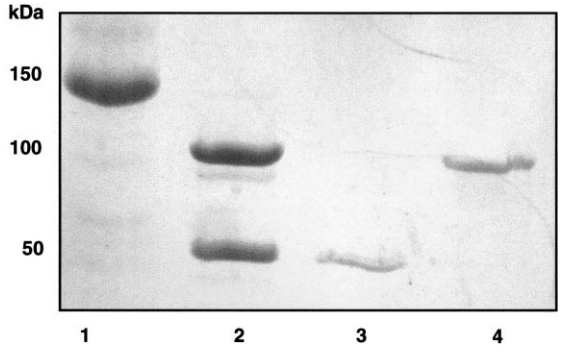

Fig. 2. SDS–PAGE analysis of BoNT/A and isolated chains. Lane 1: BoNT/A holotoxin; lane 2: BoNT/A holotoxin reduced with 50 mM dithiothreitol showing component HC (~100 kDa) and LC (~50 kDa); lane 3: purified BoNT/A LC; lane 4: purified BoNT/A HC. HC preparations were determined to be >99% pure.

3. Results

3.1. Incorporation of dyes and linker

On average, 2.3 mol of Oregon green 488 and 3.2 mol of linker were bound to each mol of dextran; 2.8 mol Cy3 was attached to each mol of HC. The coupling between HC and dextran was rapid and appeared to be complete within 5 min. This is demonstrated by SDS–PAGE in Fig. 3. Lanes 1 and 3 represent DV formed after conjugating dextran and HC for 5 and 75 min, respectively. In each case, the DV is unresolved due to variations in the molecular weights of the conjugated 10-kDa dextran. However, even at 5 min, there is no evidence of free unconjugated HC, indicating that the reaction had proceeded to completion. The incorporated HC is revealed after DV is exposed to 50 mM dithiothreitol, which reduces the disulfide that couples the dextran to the HC (lanes 2 and 4). A disulfide bond also couples the LC to HC in BoNT holotoxin (lanes 5 and 6).

3.2. Uptake of DV by cortical cells

Cortical cells exposed to 200 nM DV for 24 h showed prominent Cy3 and Oregon green 488 fluorescence as illustrated in Fig. 4A1–A3. The staining pattern of each dye was punctate and perinuclear. The punctate nature of the staining suggests clustering of DV in vesicles. Exclusion of nuclear staining indicates that the DV is intracellular, as expected for material transported by BoNT HC. Staining of soma and processes was of approximately equal intensity. This may reflect a similarity of transport sites or subsequent redistribution of DV after internalization.

The Cy3 and Oregon green staining patterns exhibited a marked degree of co-localization, which is especially apparent in the two-color overlay (Fig. 4, A3). The high level of coincidence suggests that the HC and dextran are still coupled at this stage, or that the components are confined to the same intracellular compartment.

To determine whether the presence of dextran impedes the

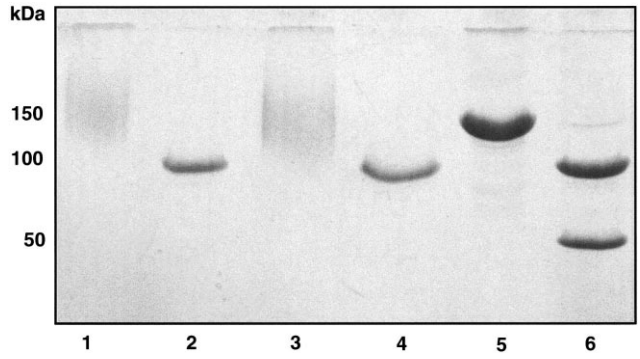

Fig. 3. SDS–PAGE analysis of DV and BoNT/A on 10–15% Phastgel stained with 0.1% Coomassie brilliant blue R250. Odd-numbered lanes correspond to unreduced DV (lanes 1 and 3) or unreduced BoNT/A holotoxin (lane 5); even-numbered lanes correspond to reduced DV (lanes 2 and 4) or reduced BoNT/A with ~100-kDa HC and ~50-kDa LC (lane 6). Reduction was performed by addition of 50 mM dithiothreitol prior to electrophoresis. HC and dextran were conjugated for 5 min in lanes 1 and 2 and for 75 min in lanes 3 and 4. Lanes 5 and 6 show BoNT/A in the absence and presence of 50 mM dithiothreitol. The complete DV is not resolved in lanes 1 and 3 due to variations in molecular weight of the 10-kDa dextran. The lack of a distinct HC band in lanes 1 and 3 indicates that coupling of HC and dextran was complete at both time points.

translocation of the HC, experiments were also performed on Cy3-labeled HC lacking the dextran moiety. Comparison of the Cy3 fluorescence of the complete DV with that of dextran-free HC revealed a similar fluorescence pattern and intensity (Fig. 4A2,B2). Thus the dextran does not appear to interfere with the internalization of the HC.

To demonstrate that the fluorescent signal represents specific intracellular transport by BoNT/A HC, cortical cells were exposed to Oregon green 488-labeled dextran under conditions identical to those used for monitoring internalization of DV. As is evident from Fig. 4C1–C3, there was no detectable Oregon green 488 fluorescence when cells were incubated with labeled dextran alone.

3.3. Competition between DV and BoNT/A

The data in Fig. 4 suggest that internalization of the DV requires the presence of the BoNT/A HC. However, it is not clear whether trafficking of DV is comparable to that of the BoNT/A holotoxin. A similarity of the internalization mechanism may be inferred by demonstrating that BoNT/A holotoxin can compete with DV. Fig. 5 shows the results of competition experiments in which cortical cultures were exposed to DV alone (A1 and B1) or to DV plus a 2-, 5-, or 10-fold molar excess of BoNT/A holotoxin. In this experiment, the Cy3 label was omitted to avoid possible alteration of the HC, and only the Oregon green 488 dextran label was present. As is clear from Fig. 5, there was a pronounced concentration-dependent reduction in the fluorescence intensity of the DV in the presence of BoNT/A holotoxin. When BoNT/A was applied at 10-fold excess (A4 and B4), the Oregon green 488 signal was no longer visible. These results suggest that DV gains entry into cortical cells by the same route as that used by BoNT/A holotoxin.

4. Discussion

The present study represents one of the first attempts to develop a targeted drug delivery system for treatment of BoNT intoxication. The need for such a system has long been recognized due to difficulties involved in directing therapeutic agents to cholinergic nerve terminals. The first report suggesting the feasibility of targeted delivery for BoNT was provided by Zhou et al. [20]. These authors showed that a catalytically inactive BoNT was able to transport the LC of tetanus toxin into cells. The current study has extended this concept by demonstrating that the HC is capable of transporting a branched polysaccharide with multiple drug binding sites into the neuronal interior (Fig. 4). For drug delivery, antagonist of BoNT LC would be attached to sites currently occupied by the Oregon green 488 reporter dye (Fig. 1).

This study also demonstrated that internalization of DV is reduced by the presence of BoNT/A, suggesting that the initial trafficking of DV is similar to that of BoNT/A holotoxin (Fig. 5). It is not clear, however, whether competition between DV and holotoxin occurs at the level of receptor binding or is exerted at subsequent steps in the internalization of the DV complex.

The targeted DV had two distinct components: the pure HC of BoNT/A and an inert dextran polymer (Fig. 1). The HC ensures that the DV will interact with cells that possess external receptors for BoNT, thus minimizing systemic toxicity of emerging BoNT antagonists [21]. The HC also promotes internalization of the attached cargo, making the BoNT antagonists available for inhibition of internalized LC. Although cortical cells are not generally involved in botulinum intoxication, they have been shown to be susceptible to BoNT as determined by fluorescent probes as well as by substrate cleavage studies [22,23].

The role of the dextran is to provide a delivery platform for reversible binding and dissociation of BoNT inhibitors. Dextrans were selected for this function because they are inert, non-toxic and stable [24]. Dextrans are hydrophilic polysaccharides characterized by their moderate to high molecular weight, good aqueous solubility and low toxicity. They are widely used as both anterograde and retrograde tracers in neurons and for numerous other applications [24]. Their uncommon poly-(D-1,6-glucose) linkage renders them resistant to cleavage by most endogenous cellular glycosidases. They also have low immunogenicity.

The 10-kDa dextran represents the first attempt at finding a suitable carrier for BoNT inhibitors. While dextrans possess a number of advantages as drug carriers, they also have an obvious limitation; dextrans contain only a small number of drug binding sites. For the 10-kDa dextran used in the current study, if drugs are coupled to the amino groups, a maximum of 3.7 mol of inhibitor can be attached for each mol of DV. Since the best BoNT antagonists currently available have K_i values in the micromolar range, it is unlikely that delivery of such a small quantity of therapeutic agents will be effective in inhibiting the proteolytic activity of the LC [15–17]. Dextrans of higher molecular weight may overcome this difficulty but at the cost of diminished targeting specificity. In preliminary studies, we examined the ability of DVs coupled to a 40-kDa amino dextran (6.1 drug binding sites per mol) to enter cultured cortical and spinal cord cells. Although the DV was able to enter both cell types, the larger dextran underwent significant internalization in the absence of HC. Other potential carriers include polyethylene glycol and polylysine, and

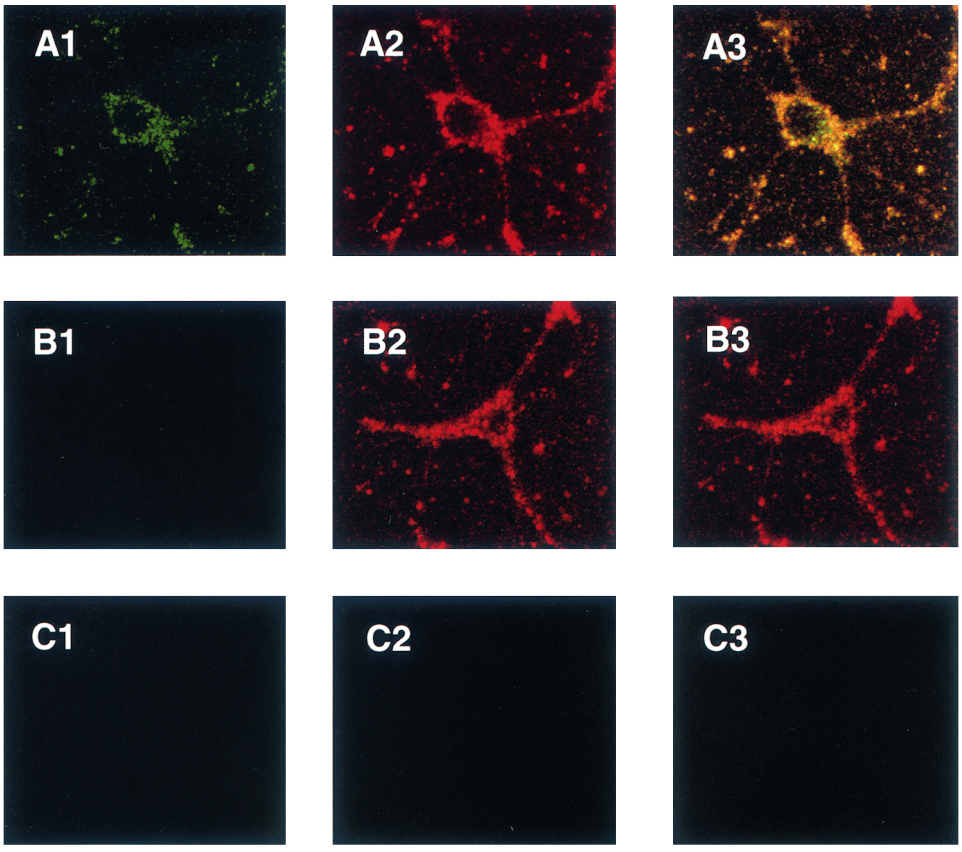

Fig. 4. Fluorescent images of cultured cortical cells. Cells were incubated for 24 h with 200 nM solutions of fluorescently labeled DV (A1–A3), HC (B1–B3) or dextran (C1–C3). Dextran, HC and DV were labeled with Oregon green 488, Cy3 or both fluorophores, respectively. The micrographs in column 1 show fluorescence elicited at an excitation wavelength of 488 nm (green); the images in column 2 were elicited at 543 nm (red). Column 3 represents overlays of the two images. Micrographs were obtained on a Zeiss LSM laser confocal microscope using a 63× oil immersion objective.

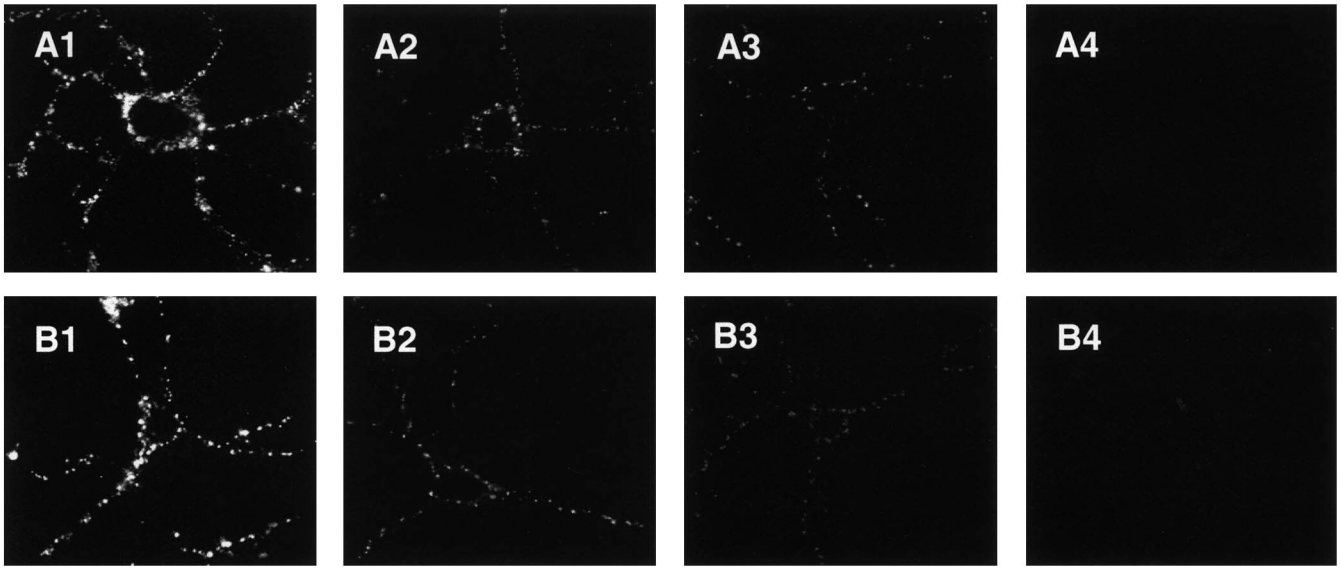

Fig. 5. Fluorescent micrographs of cortical cells demonstrating that BoNT/A holotoxin competes with DV for entry into cells. Images in rows A and B were obtained from duplicate cultures exposed for 24 h under the following conditions: (1) DV (200 nM) in the absence of BoNT/A; (2) DV (200 nM) and BoNT/A (400 nM); (3) DV (200 nM) and BoNT/A (1 μM); and (4) DV (200 nM) and BoNT/A (2 μM). DV and BoNT/A were added simultaneously. To avoid potential complications from possible Cy3-mediated alterations of HC, the latter was omitted and only Oregon green 488 was used. Note the progressive reduction in fluorescence with increasing concentrations of BoNT/A. Micrographs were obtained on a Zeiss LSM laser confocal microscope using a 63× oil immersion objective.

these will be evaluated in subsequent studies for their ability to carry a larger cargo while retaining specificity of targeting.

According to our current understanding of BoNT trafficking, the neurotoxin first binds to surface receptors and is then internalized via receptor-mediated endocytosis [1]. Initially, the toxin is confined to endocytotic vesicles; acidification of these vesicles leads to a conformational change in the toxin. The N-terminal domain of the HC then forms channels in the endocytotic vesicle membrane allowing release of the LC into the cytosol [1]. The punctate nature of the staining for DV and co-localization of the dextran and HC suggest that both components of the DV may still be located in endocytotic vesicles 24 h after incubation (Fig. 4). This finding raises the possibility that the dextran is unable to use the same pathway as the LC to escape from endocytotic vesicles. Even if the dextran remains membrane-bound, BoNT inhibitors could still be delivered to the cytosol if the drugs are membrane-permeable and are able to dissociate from the dextran under the mild acidic conditions that occur in these cellular compartments.

5. Conclusions

The present study examined the feasibility of an HC-based DV for intracellular transport of BoNT antagonists. The results indicate that the DV was capable of neuronal targeting and that entry into cells occurred by mechanisms common to BoNT holotoxin. Future efforts will focus on testing polymeric carriers with higher ligand binding capacity to allow evaluation of DV constructs coupled to actual BoNT inhibitors. Although cellular targeting strategies have not always been successful, the selectivity of BoNT HC offers cause for optimism.

References

[1] Simpson, L.L. (1989) in: Botulinum Neurotoxin and Tetanus Toxin (Simpson, L.L., Ed.), pp. 153–178, Academic Press, New York.
[2] Schantz, E. and Johnson, E.A. (1997) Perspect. Biol. Med. 40, 317–327.
[3] Shapiro, R.L., Hatheway, C. and Swerdlow, D.L. (1998) Ann. Intern. Med. 129, 221–228.
[4] Sathyamoorthy, V. and DasGupta, B.R. (1985) J. Biol. Chem. 260, 10461–10466.
[5] Montecucco, C. (1986) Trends Biochem. Sci. 11, 315–317.
[6] de Pavia, A., Poulain, B., Lawrence, G., Shone, C.C., Tauc, L. and Dolly, J.O. (1993) J. Biol. Chem. 268, 20838–20844.
[7] de Pavia, A. and Dolly, J.O. (1990) FEBS Lett. 277, 171–174.
[8] Christopher, G.W., Cieslak, T.J., Pavlin, J.A. and Eitzen Jr., E.M. (1997) J. Am. Med. Assoc. 278, 412–417.
[9] Franz, D.R., Jahrling, P.B., Frielander, A.M., McClain, D.J., Hoover, D.L. and Bryne, W.R. (1997) J. Am. Med. Assoc. 278, 399–411.
[10] Jankovic, J. and Brin, M.F. (1997) Muscle Nerve 6, S129–S145.
[11] Montecucco, C. and Schiavo, G. (1994) Mol. Microbiol. 13, 1–8.
[12] Adler, M., Nicholson, J.D., Starks, D.F., Kane, C.T., Cornille, F. and Hackley Jr., B.E. (1999) J. Appl. Toxicol. 19, S5–S11.
[13] Adler, M., Dinterman, R.E. and Wannemacher, R.W. (1997) Toxicon 35, 1089–1100.
[14] Deshpande, S.S., Sheridan, R.E. and Adler, M. (1997) Toxicon 35, 433–445.
[15] Schmidt, J.J., Stafford, R.G. and Bostian, K.A. (1998) FEBS Lett. 435, 61–64.
[16] Adler, M., Nicholson, J.D., Cornille, F. and Hackley Jr., B.E. (1998) FEBS Lett. 429, 234–238.
[17] Roques, B.P., Anne, C., Turcaud, S. and Fournie-Zaluski, M.C. (2000) Cell Biol. 92, 445–447.
[18] Goodnough, M.C. and Johnson, E.A. (1992) Appl. Environ. Microbiol. 58, 3426–3428.
[19] Matthews, C.C., Zielke, H.R., Wollack, J.B. and Fishman, P.S. (2000) J. Neurochem. 75, 1045–1052.
[20] Zhou, L., de Pavia, A., Liu, D., Aoki, R. and Dolly, J.O. (1995) Biochemistry 34, 15175–15181.
[21] Lalli, G., Herreros, J., Osborne, S.L., Montecucco, C., Rosetto, O. and Schiavo, G. (1999) J. Cell Sci. 112, 2715–2724.
[22] Verderio, C., Coco, S., Rosetto, O., Monteccuco, C. and Matteoli, M.J. (1999) Neurochemistry 73, 372–379.
[23] Adler, M., Keller, J.E., Sheridan, R.E. and Deshpande, S.S. (2001) Toxicon 39, 233–243.
[24] Vercelli, A., Repici, M., Garbossa, D. and Grimaldi, A. (2000) Brain Res. Bull. 51, 11–28.