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Human T-cell responses to botulinum neurotoxin. Responses in vitro of lymphocytes from patients with cervical dystonia and/or other movement disorders treated with BoNT/A or BoNT/B

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ABSTRACT

We have previously reported that botulinum neurotoxin type A (BoNT/A)-specific T-cell responses occur in a majority of patients treated with botulinum neurotoxins (BoNT). In this study, we first determined if T-cell responses against BoNT/A and tetanus toxin (TeNT) differ between cervical dystonia (CD) patients and other movement disorder cases. Secondly, we have examined in CD cases the treatment parameters that may have an effect on the T-cell responses against BoNT/A. We found that T-cell responses to BoNT/A were significantly higher in patients with CD than in those with other movement disorders. An increase in TeNT T-cell response in CD was observed when compared to un-treated controls. CD patients who were injected with BoNT/B mounted higher responses to BoNT/A than patients treated with BoNT/A only. Frequent injections (more than 2.1/year) were associated with a significantly higher T-cell response to BoNT/A in CD. T cell responses to BoNT/A did not differ between CD patients who had clinically responsive and non-responsive status at the time of enrollment.

1. Introduction

Cervical dystonia (CD; spasmodic torticollis) is a neurological movement disorder characterized by involuntary contractions of neck muscles resulting in abnormal neck and head movements and postures. Women are more susceptible than men, and about 5–10% of CD cases have family history of dystonia (Leube et al., 1997). The recommended treatment of CD involves injections into affected muscle (s) at 3 to 6-month intervals with a botulinum neurotoxin type A (BoNT/A) or BoNT type B (BoNT/B) preparation (complex of BoNT and hemagglutinin) (Jankovic, 2004; Simpson et al., 2008). Repeated injections, however, may lead to antibody (Ab) responses against the immunizing toxin (more frequent with BoNT/B than with BoNT/A) (Göschel et al., 1997; Jankovic, 2002; Atassi, 2004; Jankovic et al., 2006). In some patients these Ab responses have neutralizing activity (i.e., blocking Abs) against the toxin and prevent the patient's responsiveness to further treatment (Göschel et al., 1997; Naumann et al., 1998; Jankovic, 2002; Atassi, 2004; Lange et al., 2009). When immunoresistance occurs an alternative toxin serotype is often used (usually BoNT/A is switched to BoNT/B) (Sloop et al., 1997; Dressler et al., 2003; Comella et al., 2005). Within a few injections with BoNT/B, however, blocking Abs often appear and cause non-responsiveness and therapy failure (Dressler and Bigalke, 2004).

In the preceding paper (Oshima et al., 2011) we reported that specific anti-BoNT/A T-cell responses appear in peripheral blood lymphocytes (PBL) of about 70% of patients with CD or other movement disorders (termed non-CD) during treatment with BoNT. The finding provides an important base on understanding how T-cell mediated immunity against BoNT develops in the treatment process with BoNT. Our observation also may have an implication for a possible new immunological mechanism: in which unconventional conditions such as prolonged but minute dose toxin injections might develop T-cell immunity without activation of immune responses of IgG Ab-producing B cells. The T-cell responses

Abbreviations: Ab, antibody; BoNT/A, botulinum neurotoxin type A; BoNT/B, botulinum neurotoxin type B; CD, cervical dystonia; ECD, exclusive CD (i.e., patient diagnosed with CD only); FET, Fisher's exact test; MPA, mouse protection assay; non-CD, other movement disorders; NECD, non-ECD (i.e., patient diagnosed with CD plus other movement disorders); PBL, peripheral blood lymphocytes; SI, stimulation index (cpm of ^{3}H-thymidine incorporated by antigen-stimulated T cells/cpm incorporated by unstimulated cells); TeNT, tetanus toxin or tetanus neurotoxin; *T* test, Student's *T* test.

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to BoNT/A were similar regardless of the responsiveness status, at the enrollment to this study, of the patients to the treatment. An increase in the T-cell response of BoNT-treated patients to tetanus neurotoxin (TeNT) was also observed. Although blocking Abs have been frequent focuses in immunological studies in BoNT-treated CD, T-cell responses against BoNT that help B-cell development have never been studied before for this disease. Examination of the influences on the T-cell responses of treatment-responsiveness status or of treatment parameters is also essential for understanding the development process of T-cell immunity resulting from BoNT treatment. In this report we have briefly examined the differences in T-cell response patterns between CD and non-CD, and between exclusive CD (ECD) and remaining non-CD-associated cases. We then analyzed correlation between T-cell response and responsiveness status to BoNT treatment of CD patients and treatment parameters that may determine the T-cell responses of CD. For controls, we used PBL from normal healthy individuals and patients with CD and/or other neuromuscular disease who were never injected with BoNT.

2. Materials and methods

2.1. Inactivated toxins and blood samples

Inactivated BoNT/A and TeNT were obtained from the same sources and prepared for in vitro cell challenge as described (Oshima et al., 2011).

Blood samples were obtained from patients with the clinical diagnosis of CD (n = 80) or of other movement disorders (termed non-CD) (n = 15) who have been treated with BoNT/A (original BOTOX® before 1998 and/or current BOTOX® afterwards, Allergan) and/or BoNT/B (Myobloc®, Solstice Neurosciences) injections. Patients were injected by 3 physicians at 2 clinics. Details on use of BoNT/A and BoNT/B during the course of treatments are as follows: among 80 CD patients, 34 received original BOTOX® followed by current BOTOX®, of which 19 also received BoNT/B injections. While the remaining 46 received current BOTOX®, of which 6 also received BoNT/B and 2 received BoNT/A of other brands, Dysport® (Ipsen Biopharm; n = 1) and Xeomin® (Merz Pharma GmbH; n = 1), in addition to BOTOX®. Among 15 non-CD patients, 7 received both forms (4 cases with BoNT/B) and 8 received current form of BOTOX® (0 case with BoNT/B but 1 case with Dysport® use). CD was evaluated by the Toronto Western Spasmodic Torticollis Scale (TWSTRS) (Comella et al., 1997). Blocking Abs in the sera were confirmed in mice by the mouse protection assay (MPA) as described (Atassi et al., 2005). Control samples (n = 63) were obtained from patients with CD (n = 2) and with other neurological diseases (n = 40) and normal healthy individuals (n = 21) who had never received BoNT injections. Demographic information on CD and non-CD patients as well as control subjects is summarized in Table 1. Informed written consent, approved by the Baylor College of Medicine Institutional Review Board for Human Research, was obtained from patients at their enrollments.

Diagnoses of 15 non-CD cases, in which 5 patients had two diagnoses each, included: other forms of dystonia (n = 10), 2 forms of spasm (4), essential tremor (1), peripherally induced post traumatic (1), Tourettes syndrome (1), Parkinson's disease (1), post-anoxic encephalopathy (1) and tardive dyskinesia (1). Of the 80 CD cases, 62 were diagnosed with CD only (termed exclusive CD; ECD), while the remaining 18 were diagnosed with CD and co-existing other movement disorders (termed NECD). Diagnosis for non-CD in the latter group included: other forms of dystonia (n = 10), essential tremor (5), peripherally induced post traumatic (2) and Tourettes syndrome (1). Treatment information comparing CD versus non-CD, and ECD versus NECD + non-CD was listed in Table 2. Dose (in units of biologic activity) of BoNT/B was converted as approximate dose of BoNT/A (BOTOX®) (BoNT/B 50 U = BOTOX® 1 U) in this study. Also, doses of Dysport® and Xeomin® were converted as approximate dose of BOTOX® (2.5 U of Dysport® = 1 U of BOTOX®; 1 U of Xeomin® = 1 U of BOTOX®). These conversion ratios have been chosen based on most comparative studies and the physicians' personal experience that the stated ratios provide equivalent clinical results and same injection volumes.

Table 1
Demographic information of CD and non-CD cases and control subjects.

	CD	Non-CD	Control
	N = 80	N = 15	N = 63
Race:			
Caucasian	73	12	43
African-American	1	3	4
Hispanic	5	0	9
Asian	1	0	7
Age (year; average, range)	60 (31–90)	59 (21–79)	50 (16–77)
Gender:			
Female	57	9	28
Male	23	6	35
Onset:			
Early (<30 years)	11	4	
Late	69	11	
Family history of movement disorders:			
Yes	33	5	
No	47	10	
TWSTRS score[a] (average, range):			
Severity (max = 35)	18.7 ± 6.8 (5–29)		
Disability (max = 30)	14.6 ± 7.4 (0–30)		
Pain (max = 20)	10.4 ± 6.2 (0–20)		
Total (max = 85)	42.3 ± 15.0 (7–72)		

[a] TWSTRS scores are from 44 CD patients.

2.2. Isolation of peripheral blood lymphocytes (PBL) and proliferation assay

PBLs were isolated from heparinized blood samples as described (Oshima et al., 2011). PBLs from BoNT-treated patients and un-treated controls were co-cultured in triplicates in 96-well flat-bottom microtiter plates (Corning-Costar) at 5×10^5 cells/well in a final volume of 200 μl or 2.5×10^5 cells/well in a final volume of 100 μl of IMDM medium (Invitrogen, Grand Island, NY) containing 5% normal (type AB) human serum and various concentrations (5–20 μg/ml) of inactivated BoNT/A and TeNT. In addition to unstimulated cells, controls included cells stimulated with PHA-P (10 μg/ml), Con A (2 μg/ml) or control proteins (lysozyme, OVA; 100 μg/ml). After incubation for 3–4 days at 37 °C in a humidified CO_2 atmosphere, the cultures were pulsed with [^{3}H]dThd (1 μCi/well = 37 kBq)(MP Biomedicals, Irvine, CA), harvested onto glass microfiber filters and the radioactivity counted by liquid scintillation as described (Oshima et al., 2011). The results are expressed as mean cpm of [^{3}H]dThd uptake or as stimulation index (SI; SI = cpm incorporated by stimulated cells/cpm incorporated by unstimulated cells). For the purpose of this study, an SI value > 2.0 is considered as a positive response after reviewing responses to BoNT/A of normal healthy controls (n = 13; SI ranged 0.92 to 1.81; mean SI, 1.23 ± 0.25; SI + 3SD ≈ 2.0) that had no response to TeNT.

2.3. Statistical analyses

T-cell responses to BoNT/A or TeNT of treated and control groups showed skewed distributions and had marked kurtosis. Therefore, results of the T-cell proliferative assays were expressed as SI ± SD as well as transformed SI obtained by converting SI values to the log scores. The geometric mean (antilog of mean of transformed values) and antilog of the transformed 1SD boundaries (minus the geometric mean) are also presented in the figure legends. BoNT-treated patient samples are grouped according to clinical diagnosis (CD or non-CD; CD or NECD; or ECD or NECD + non-CD). CD patient samples were further grouped according to responsiveness status (at the