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Development of inhibition ELISA to detect antibody-induced failure of botulinum toxin a therapy in cosmetic indications

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ABSTRACT

Secondary treatment failure (STF) of botulinum toxin A (BoNT/A) therapy in cosmetic indication has been postulated as production of antibody against active sites of BoNT/A in unresponsive patients. To prove of concept, detection of anti-BoNT/A antibody is required, however, current enzyme-linked immunosorbent assay (ELISA) detects human IgGs against whole BoNT/A molecule. We developed an inhibition ELISA to quantify antibodies bound to the active sites of BoNT/A using three mouse monoclonal antibodies targeting translocation domain, receptor binding site and catalytic domain of BoNT/A prior to processing ELISA to detect human IgG (hIgG) against BoNT/A. Adults naïve to BoNT/A, or treated and responsive (toxin-response), or treated but unresponsive (toxin-tolerance) were recruited. Detection of hIgG revealed that naïve volunteers had basal level of hIgG against whole BoNT/A, whereas its level was significantly lower than those hIgG in BoNT/A-exposed cohorts. Higher anti-BoNT/A levels in sera from volunteers ever-exposed to BoNT/A indicates that BoNT/A may provoke immune responses in BoNT/A-treated cohorts. Inhibition ELISA demonstrated that levels of BoNT/A-specific hIgG in tolerance patients had a dramatic decrease in mouse monoclonal antibody blockage, suggesting presence of hIgG specific to BoNT/A's three active sites in STF patients. Therefore, our ELISA detected hIgG against whole BoNT/A protein and BoNT/A active sites suggesting that human antibodies may cause STF. To compare with frontalis test, our inhibition ELISA provided good accuracy at 83.1% (50% sensitivity and 89.9% specificity). Our test may help clinicians to diagnose possibility of STF and also to monitor immune status against BoNT/A.

1. Introduction

In 2017, over 7 million injections of botulinum toxin A (BoNT/A) were delivered for cosmetic indications, 819% increase from just under 800,000 in 2000 and 2% from 2016 (American Society of Plastic Surgeons, 2017). Due to its proven efficacy, versatility and safety, BoNT/A is also applied in the clinical treatment of hypersecretion, ophthalmology, urology, gastrointestinal systems and pain disorders. However, the use of BoNT/A often results in secondary treatment failure. Such events have been proposed to relate with the increasing usage frequency or injection volumes of BoNT/A, but not yet proven, also are worrying for physicians seeking to achieve their patients' requested outcomes.

Some patients may require multiple treatments and injections over an extended period, thus making the use of inexpensive toxins seem ideal (Torres et al., 2014). With all other treatment factors being equal (e.g. injection depth), this strategy is only logical if the chosen toxin is consistently and completely effective at each subsequent treatment session, and if the toxin used is guaranteed not to induce immune responses. However, all botulinum toxins are foreign proteins, and some commercial preparations contain additional non-toxin or complexing proteins that may also act as foreign antigens to induce production of antibodies. Such antibodies can be associated with secondary treatment failure, particularly with multiple injections (Benecke, 2012; Dressler, 2002). Immunogenic or antigenic foreign proteins include structural proteins, hemagglutinin and non-hemagglutinin proteins, peripheral or neurotoxin-associated proteins (NAPs), and non-toxin, non-hemagglutinin proteins can provoke immune response leading to antibody-induced botulinum treatment failure (ABTF) (Dressler et al., 2018). Of the commercially-available botulinum toxins approved by the U.S. FDA for use in glabellar frown lines, incobotulinum toxin A (incoA) remains the only product that is purified form with low antigenicity and could be alternative for ABTF (Dressler et al., 2018; Merz Pharma GmbH and Co KGaA, 2011).

Although sparsely documented, there is evidence of immune reactions targeting BoNT/A and resulting in treatment failures. A single case series presentation of five patients, who were each treated with low toxin doses by independent physicians, showed that all patients tested positive for neutralizing antibodies to BoNT/A (nAb) (Torres et al., 2014). All patients had toxin delivered to multiple injection sites of the

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upper face. A meta-analysis also found that of 11 patients who had nAbs to onaA, 3 patients became non-responsive and failed treatment (Naumann et al., 2010). As others have noted, without clinical trials to directly evaluate and compare nAb levels induced by different BoNT/A formulations, it is difficult to conclude definitively whether BoNT/A immunogenicity induces significant nAb responses (Dover et al., 2018).

Although only 0.3–6% of patients are thought to develop nAbs, and some studies failed to find evidence of nAbs, the many emerging, small-scale reports of secondary treatment failures and non-responsiveness suggest that much better and more conclusive nAb detection methods are needed (Brandt et al., 2009; Lawrence and Moy, 2009; Monheit et al., 2009; Moy et al., 2009). Currently, mouse protection assay and mouse phrenic nerve hemidiaphragm assay are the tests used to imply the presence of nAbs in serum sample that they could protect mice from lethal doses of BoNT/A administered into the mice (Buelbring, 1946; Goeschel et al., 1997; Pearce et al., 1994). However, these tests are not only unethical due to the need for large quantities of animals, but they also waste time and resources, which are impractical for use in daily clinical practice or to quickly diagnose the cause of treatment failure (Pellett, 2013).

As an alternative to animal model-based detection methods, Dressler and colleagues ever developed in vitro test to detect human anti-botulinum toxin antibody by ELISA technique (Dressler et al., 2014). Their technique just detected antibody against whole molecule of BoNT/A, which could not be implied to explain neutralization of antibody against BoNT/A and lack of good control of test. Therefore, our study aimed to develop in vitro, highly-sensitive enzyme-linked immunosorbent assay (ELISA) with modification. This ELISA aims to detect and quantify antibodies bound to the active epitope of BoNT/A in patient sera, that occur as a consequence of secondary treatment failure.

2. Materials and methods

2.1. Experimental design

This prospective cohort study was conducted in a single centre. Healthy adults who had never received BoNT/A treatments (naïve), or had received BoNT/A and were responding (toxin-responsive), or had received BoNT/A but did not respond to it (toxin-tolerant), were recruited. Toxin-treated patients were subjected to the frontalis test prior to inclusion or exclusion. BoNT/A was unilaterally injected into frontalis muscles as previously described (Marion et al., 2016). Briefly, toxin was administered in two injections 3 cm above the lateral and medial canthus of one eye but not the contralateral eye. Eyebrow-raising abilities were evaluated after 2–4 weeks, with asymmetric muscular reactions showing that BoNT/A was effective. Toxin-tolerant patients were identified through non-responsiveness to toxin administered during the frontalis test.

2.2. Patient sample collection and preparation for ELISA

Ten milliliters of blood were collected from patients two weeks after the frontalis test to facilitate time for result analysis. The blood samples were centrifuged at 1000 rpm for 10 min at room temperature. Sera in supernatants were kept at -20 °C until use.

2.3. ELISA to detect human anti-botulinum toxin antibody

A 96-well ELISA plate was coated overnight at 4 °C with 0.014 unit/ml of commercial incobotulinum toxin A (incoA; Xeomin®) solution, and 0.25 to 0.0019 µg/ml of human immunoglobulin G (hIgG) was also used to coat wells to establish a standard curve. After three washes, non-specific binding was blocked with 1%-BSA at 37 °C for 1 h. Following three washes, 50 µl of 1:50 diluted serum samples were added to incoA-coated wells and incubated at 37 °C for 2 h. Following 5 washes, freshly-prepared, HRP-conjugated rabbit anti-hIgG solution was added to all sample wells. IncoA-positive (incoA coated) controls were detected with rabbit polyclonal anti-BoNT/A antibody. hIgG standard curve wells (positive control) and hIgG negative (no hIgG coating) controls were detected with HRP-conjugated rabbit anti-hIgG antibody. Tetramethylbenzidine was used as color development substrate and optical density (OD) was measured at 450 nm with an ELISA plate reader.

2.4. Inhibition ELISA

Briefly, a mixture of three commercial mouse monoclonal antibodies (1:1:1 ratio) targeting the incoA translocation domain, receptor binding site (clones 2A33 and 24A29, respectively) and catalytic domain (clone 503013) was added to ELISA plates prior to adding diluted sera. Mixture of mouse monoclonal antibodies was allowed binding to incoA for 2 h at 37 °C. After five time washing, diluted sera were added and ELISA to detect human anti-BoNT/A IgG was perfromed as previously described.

2.5. Statistical calculations

Either parametric (*t*-test or paired t-test) or nonparametric (sign test or signed-rank test) was used as appropriate. A *p*-value of < 0.05 was considered to be statistically significant. For accuracy and consistency of data interpretation, raw values from inhibitory ELISA experiments were normalized. To calculate the level of reduction in antibody-antigen binding, a percentage of inhibition was derived through dividing the difference between values for non-inhibited and inhibited samples with the values for the non-inhibited sample. Threshold values were obtained by applying receiver operating characteristic (ROC) data analysis. Validation of inhibitory ELISA was performed against 14 toxin-tolerant samples and 69 control samples (naïve and toxin-responsive). Values for sensitivity, specificity, positive and negative predictability (PPV and NPV, respectively), and accuracy were then calculated.

2.6. Ethical approvals

This study was approved by the Ethical Committee on Research Involving Human Subjects, Faculty of Medicine, Siriraj Hospital, Mahidol University (COA No. Si 540/2016), and conformed to the guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from all study subjects.

3. Results

3.1. Patient demographics

Eighty-three volunteers were recruited (Table 1). 35 naïve (25 females and 10 males, mean age 29.2 years), 22 responsive (19 females and 3 males, mean age 39.5 years) and 28 tolerant patients, (23 females and 5 males, mean age 32.5 years) segregated by frontalis testing. The detailed demographic and clinical data of patients in each group were described in Table 1. When comparing between responsive and tolerant group, dose per visit and total dose of BoNT/A receiving for cosmetic indications were significant different. The tolerant group tended to receive higher dose per visits and total cumulative dose prior to the study.

3.2. Detection of human antibody specific to whole BoNT/a molecule

Naïve patients demonstrated basal levels of human IgG (hIgG) against the whole BoNT/A molecule (Fig. 1), even though they had never been exposed to therapeutic BoNT/A. In comparison, BoNT/A-exposed cohorts (responsive and tolerant) displayed significantly higher

Table 1
Patient demographics and clinical data.

Groups	Average Age ± SD	Sex		Median duration (years)	Median dose / visit (units)	Median number of Injection	Median interval (months)	Median total dose (units)
		Female, n (%)	Male, n (%)					
Naive	29.2 ± 7.1	25 (73.5)	10 (26.5)	–	–	–	–	–
Responsive	39.5 ± 12.3	19 (86.4)	3 (13.6)	5	50	8	6	375
Tolerance	32.6 ± 5.4	23 (82.1)	5 (17.9)	7	100	10	6	775
P value (*: p < .05)	0.547	0.447	0.109	0.420	0.003*	0.215	0.252	0.020*

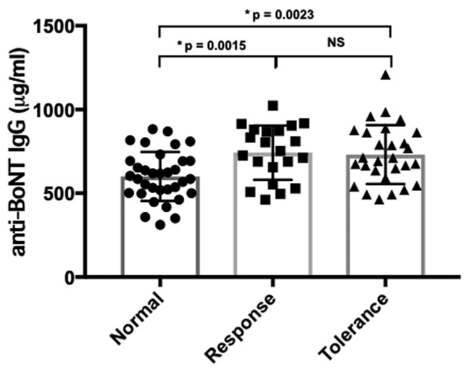

Fig. 1. Overlay of individual ELISA datapoints. Bar graphs represent levels of human IgG against incobotulinum toxin A in naïve, toxin-responsive and toxin-tolerant cohorts (Mean ± SEM), as detected in ELISA plates pre-coated with the relevant toxin. Scatter plots of individual patient data points were overlaid onto bar graphs representing levels of human IgG binding to BoNT/A in naïve, toxin-responsive and toxin-tolerant cohorts (Mean ± SEM).

($p < .05$) levels of anti-BoNT/A hIgG.

3.3. Inhibition ELISA detection of human antibody specific to three BoNT/A active sites

Anti-BoNT/A hIgG levels within the blocked mouse monoclonal antibody (mMAb) mixture indicated the amount of anti-BoNT/A hIgG against the toxin's apart from three active sites (Fig. 2). hIgG specific to BoNT/A non-active sites were compared to hIgG against the whole BoNT/A molecule (Fig. 2a). Anti-BoNT/A hIgG levels following mMab ('post-blockage') significantly decreased in tolerant patient sera ($p < .0001$). Match-paired results also revealed a dramatic decrease in BoNT/A-specific hIgG in tolerant patients suggesting the presence of human IgG specific to three active sites of BoNT/A in the sera of secondary treatment failure patients (Fig. 2b).

3.4. Validation of inhibition ELISA

Our test relies on decreasing BoNT/A-specific hIgG levels indicating the hIgG levels against BoNT/A's three active sites. To normalize the differences in basal levels, decreasing values were converted into percentages of inhibition and analyzed by receiver operating characteristic (ROC) analysis. ROC analysis yield sensitivity and sensitivity of each percentages of inhibition in trial population for further validation. To obtain cut-off point for percentage of inhibition, Youden's index derived from sensitivity and sensitivity of each percentages of inhibition was used to suggest optimal cut-off point. The highest value of Youden's index at 106.8 was chosen to achieve an optimal cut-off value of 4.4 (Table 2). After validating this cut-off with additional 83 patients' serum, sensitivity of this test was found to be at 50.0%, specificity was 89.9%, PPV was 50.0% and NPV was 89.9%, giving our inhibition ELISA assays an accuracy of 83.1%.

4. Discussion

Our study demonstrated that high dose per visit and cumulative dose associated with secondary treatment failure, indicating that chance of botulinum toxin tolerance could be induced by more amount of received BoNT/A. This finding could be informative to suggest aesthetic physician to monitor how amount of BoNT/A that patients exposed. By the way, it might not be easy to follow track record of BoNT/A consumption by each patient. As we have proposed that BoNT/A-induced immune response causing secondary treatment failure, one way to overcome such issue is to monitor a patient's immune-reactivity to BoNT/A before product selection. Our simple ELISA detected human anti-toxin antibodies in all sera, which was unsurprising since the BoNT/A molecule can be epitopically similar to the tetanus toxin. Thus, human anti-tetanus antibodies can be present at basal levels in toxin-

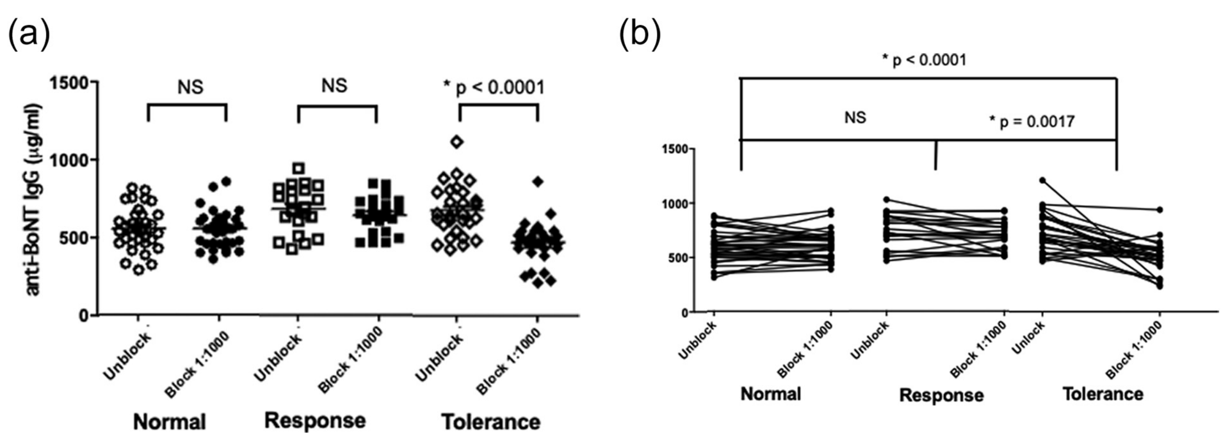

Fig. 2. Inhibition ELISA to detect hIgG against active sites of BoNT/A. (a) Scatter plot with mean values are shown and represent levels of human IgG against BoNT/A in naïve (circle), toxin-responsive (square) and toxin-tolerant (rectangle) groups before (open symbols) and after active site blockage or inhibition (closed symbols). (b) Match paired line graphs represent levels of human IgG against BoNT/A in naïve, toxin-responsive and toxin-tolerant groups before and after active site blockage.

Table 2
Cut-off values determined using Youden's index calculations.

Cut off value (percentage of inhibition)"	Sensitivity	Specificity	Youden's index
> 1.000	20.51	79.71	99.2
> 1.450	20.51	81.16	100.7
> 1.950	20.51	82.61	102.1
> 2.450	20.51	84.06	103.6
> 2.700	20.51	85.51	105.0
> 3.300	17.95	85.51	102.5
> 3.900	17.95	86.96	103.9
> 4.050	17.95	88.41	105.4
> 4.400	17.95	89.86	106.8
> 4.750	15.38	89.86	104.2
> 5.800	15.38	91.3	105.7
> 7.500	12.82	91.3	103.1
> 8.700	10.26	91.3	100.6
> 9.650	7.692	91.3	98.0

naïve patients and recognize BoNT/A. We tested tetanus toxoid-coated ELISA plates with all sera and detected the human anti-tetanus toxoid IgG (unpublished data). Anti-BoNT/A antibody levels were significantly higher in sera from volunteers ever-exposed to BoNT/A, suggesting that BoNT exposure could provoke immune responses in BoNT/A-treated cohorts. Our finding agreed with those of the only other published BoNT/A ELISA (Dressler et al., 2014). To investigate the recognition specificity of human anti-BoNT/A antibodies, our inhibition ELISA used three mMAb clones specific to three BoNT/A active sites, which were used to rescue mice in mouse assays (Smith et al., 2005). mMAb blockage revealed significant levels of human antibodies to BoNT/A active sites only in tolerant patients. To circumvent issues with mMAbs cross-reacting with tetanus toxin, plates coated with tetanus toxoids were tested identically to previous protocol. No significant decrease in hIgG bound to tetanus toxoid was found (unpublished data), suggesting that hIgGs specific to the three BoNT/A active sites correspond with BoNT/A-linked treatment failure. The previous ELISA detects and partially quantifies anti-BoNT/A antibodies, and was highly-sensitive and highly-specific for BoNT/A (Dressler et al., 2014). However, it was developed using only 30 serum samples, whereas we used over 30 sera per cohort and based our results on autologous frontalis testing. Our ELISA also contained an internal control (incoA-positive) and a standard curve that was applicable to inter-ELISA comparisons. Our inhibitory ELISA demonstrated antibody binding to three active sites potentially associated with treatment failure, and was validated with sera from an additional 14 positive frontalis test patients and 69 control patients (negative frontalis test and naïve patients). Although inhibition ELISA has poor sensitivity, it has excellent specificity and accuracy, which clinicians may exploit to establish immune statuses after BoNT/A treatment during follow-up.

In conclusion, our ELISA detects hIgG against whole BoNT/A protein and BoNT/A active sites, which indicate the presence of human anti-BoNT/A antibodies. These proof-of-concept results demonstrate that anti-BoNT/A active site antibodies can cause secondary treatment failure. Importantly, our ELISA enables clinicians to monitor a patient's immune status against BoNT/A at follow-up and may help them to choose the most appropriate BoNT/A subsequently. Our tests are ideal for surveying the immunological responses of patients to BoNT/A and help physicians achieve optimal outcomes.

Author contributions

YS and RW conceived and designed the experiments; WK and RC performed the experiments; WK, RC, PS and PT prepared the data; YS and RW analyzed the data; recruited patients; YS and RW wrote the paper with assistant cooperation from Merz Asia Pacific, Singapore.

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The authors declare no conflicts of interests with this study.

Declaration of Competing Interest

The authors declare no conflict of interest. The funding sponsors had no role in either the design of the study, the collection, analysis and interpretation of the data, the writing of the manuscript or the decision to publish the results.

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