Comparison of mouse bioassay and immunoprecipitation assay for botulinum toxin antibodies

Philip A Hanna, Joseph Jankovic, Angela Vincent

Abstract

***Objective*—To compare a recently developed immunoprecipitation assay (IPA) to the mouse protection bioassay (MPB), currently considered the "gold standard", for detecting antibodies against botulinum toxin A (BTX-A) and to correlate these assay results with clinical responses to BTX-A injections.**

***Methods*—MPB and IPA assays were performed on serum samples from 83 patients (38 non-responders, 45 responders) who received BTX-A injections. Six non-responders had serum tested on two separate occasions. Some patients also received a "test" injection into either the right eyebrow (n=29) or right frontalis (n=19).**

***Results*—All patients antibody positive (Ab+) by MPB were also Ab+ by IPA, whereas an additional 19 patients (17 with reduced or no clinical response) who were MPB Ab− were Ab+, with low titres, by IPA. Two of these 19 patients (non-responders) were initially MPB Ab− but later became MPB Ab+. Similar to previous studies, the sensitivity for the MPB was low; 50% for clinical, 38% for eyebrow, and 30% for frontalis responses whereas the IPA sensitivity was much higher at 84% for clinical ($p<0.001$), 77% for eyebrow ($p=0.111$, NS) and 90% for frontalis responses ($p<0.02$). The IPA specificity was 89% for clinical, 81% for eyebrow, and 89% for frontalis responses, whereas the MPB specificity was 100% for all three response types, which were all non-significant differences.**

***Conclusions*—Both assays had high specificity although the sensitivity of the IPA was higher than the MPB. In addition, the IPA seems to display positivity earlier than the MPB, and as such, it may prognosticate future non-responsiveness. Eyebrow and frontalis "test" injections correlated well with clinical and immunological results and are useful in the assessment of BTX non-responders.**

(*J Neurol Neurosurg Psychiatry* 1999;**66**:612–616)

Keywords: dystonia; botulinum toxin; antibodies; immunoresistance

Parkinson's Disease Center and Movement Disorders Clinic, Department of Neurology, Baylor College of Medicine, Houston, Texas, USA
P A Hanna
J Jankovic

Neurosciences Group, Department of Clinical Neurology, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford UK
A Vincent

Correspondence to:
Dr Joseph Jankovic, Department of Neurology, Director of Parkinson's Disease Center, and Movement Disorders Clinic, Baylor College of Medicine, 6550 Fannin St No 1801, Houston, Texas 77030, USA.
Telephone 001 713 798 5998; fax 001 713 798 6808.

Received 27 August 1998
and in revised form
16 November 1998
Accepted 26 November 1998

An ever increasing number of disorders including dystonia, tremors, tics, hemifacial spasm, spasticity, sphincter dyssynergia, and achalasia are now being treated effectively with botulinum toxin type A (BTX-A).[1–10] In addition, BTX-A is also being used for various non-neurological (for example, cosmetic) indications. As the range of uses for BTX-A continues to expand, there is a growing concern regarding the development of immunoresistance secondary to blocking antibodies (Ab).[2 11] The reported frequency of such antibodies has ranged from 3% to 57% depending on the assay method used.[12 13] The standard assay for detecting BTX Ab is the in vivo mouse protection bioassay (MPB), which evaluates the ability of increasing dilutions of a patient's serum to protect mice from lethal doses of BTX-A.[14] In vitro assays, including the sphere linked immunodiagnostic assay (SLIDA),[13] enzyme linked immunosorbent assay (ELISA),[15 16] a monoclonal antibody based immunoassay,[17] and western blot technique[18] have also been reported to detect such antibodies. These assays, however, do not correlate well with clinical responses because they do not detect specific blocking Ab.

The MPB has been shown to have high specificity, but its sensitivity is relatively low.[18] The primary aim of this study was to compare the MPB with a more recent immunoprecipitation assay (IPA) developed by Palace *et al*[19] and to correlate the presence of antibodies detected by these two assays to the patients' clinical response to BTX-A injections. The results described by Palace *et al*[19] needed to be confirmed using a larger number of patients, as well as incorporating more clinical details including correlation with facial (eyebrow and frontalis) "test" injections. Additionally, we evaluated the utility of eyebrow or frontalis injections[18] as clinical "tests" for immunoresistance.

Methods

Eighty three patients (17 men and 66 women) with a mean age of 56 (SD 12.2) years: range 19 to 81) were selected for this study. Most of the patients were treated primarily for dystonia; cervical (n=62; 32 non-responders), cranial and cervical (n=10; four non-responders), and cranial (n=7, all responders). Other conditions included spastic hemiplegia (n=1; responder), hemifacial spasm (n=1; responder), focal leg dystonia (n=1; non-responder), and segmental myoclonus (n=1; non-responder). Clinical response to BTX-A (Botox®, Allergan Pharmaceuticals, Irvine, CA, USA) injections was rated on a 0 to 4 "peak effect" scale (0=no effect; 1=mild effect, no functional improvement; 2=moderate improvement, no change in

functional disability; 3=moderate change in severity and function; 4=marked improvement in severity and function).[20] There were 38 non-responders (0 or 1 "peak effect" response rating after their last injection), and 45 responders randomly chosen from the botulinum toxin clinic population. Six patients (all non-responders) had samples drawn on two occasions (with a minimal latency between sample collections of 4 months; mean 4.3 months). Thus, there were 89 total serum samples on 83 patients included in this study. This low ratio of responders/non-responders does not represent the actual patient response rate in the BTX clinic as we did not collect samples on all patients seen in the clinic.

The blood collected was separated and sent to Northview Pacific Laboratories, Berkeley, California, USA for the MPB assay and to the Institute of Molecular Medicine, John Radcliffe Hospital, Oxford University, UK for the IPA assay. The individual laboratories were "blinded" to the clinical information before sample testing to maintain objectivity.

The MPB is a qualitative test reported as either positive (Ab+) indicating that the patient's serum neutralises the effects of BTX-A injected intraperitoneally with survival of 3/4 mice.[14] In a negative result, two or more of the mice die, presumably indicating the lack of blocking Ab in the patient's serum.

The IPA method was performed as described by Palace *et al*[19] with slight modifications. After the iodination reaction,[19] the ^{125}I-BTX was microfiltered and stored at 4°C in phosphate buffered saline (PBS). When required, it was diluted in PBS and centrifuged to remove any aggregates immediately before use. Supernatant (25 µl) containing 30 000–50 000 cpm, was incubated with 2.5 µl of each serum in a total volume of 50 µl PTX buffer PBS (0.02M phosphate, pH 7.4 m 0.1% triton×100). After 2 hours at room temperature or overnight at 4°C excess goat antihuman Ig was added. When a precipitate had formed, 600 µl PTX was added before centrifuging. The pellets were washed twice briefly in PTX and counted on a Cobra Packard gamma counter. Results were expressed as pM (pmoles of ^{125}I-BTX precipitated/l serum) after subtraction of the mean results (<1000 cpm) from healthy control serum samples run in parallel.

Twenty nine patients were also injected with a test dose of 15 units (n=26) to 20 units (n=3) of BTX-A into the medial aspect of their right eyebrow (one site), and 19 patients received 15 units (in two divided doses) into the frontalis muscle on the right side.[18] For the eyebrow injections, a positive (good) response is indicated by the presence of asymmetry on frowning after unilateral BTX-A injections, whereas a negative (no) response (immunoresistance) is present when there is no asymmetry with frowning. A positive response to unilateral frontalis injections is strongly suggested by asymmetry of eyebrow elevation or forehead wrinkling on raising of eyebrows whereas a symmetric contraction of forehead muscles indicates a negative response (immunoresistance).[18]

Sensitivity, specificity, and positive predictive value of the two assays was determined as follows:

Sensitivity=A/(A+C); specificity=D/(D+B); positive predictive value=(PPV) A/(A+B); negative predictive value=(NPV): D/(D+C)

where A=true positive (Ab+ with negative response to injection), B=false positive (Ab+ with positive response to injection), C=false negative (Ab− with negative response to injection), D=true negative (Ab− with positive response to injection).

Comparisons of the above parameters of the two assays were performed using the Fisher's exact test.

Results

The distribution of results of the first samples on the 83 patients is shown in fig 1. The threshold for positivity, 50 pM of ^{125}I-BTX binding sites precipitated/l of serum, was lower than that reported previously[19] due to slight improvements in the assay that reduced non-specific precipitation by control serum samples.

There was a clear correlation between the results of the IPA and MPB assays (fig 2). All serum samples which were Ab+ by MPB were Ab+ by IPA, and all Ab− samples by IPA were Ab−by MPB. However, 20 serum samples (from 19 patients) were Ab−by MPB but Ab+ by IPA. The antibody titres in this group, with a mean of 183.2 pM (SD 111.8): range 51 to 459 pM) were, however, significantly lower ($p<0.0001$, Kruskal-Wallis test) than those in the MPB Ab+ group, in which the mean was 1378.1 (SD 921.5): range 101 to 3663 pM). Of the 19 IPA Ab+/MPB Ab− patients, 14 were non-responders and two of these non-responders became Ab+ by MPB on repeat testing as shown in figure 2. The remaining five were considered false positive as they contin-

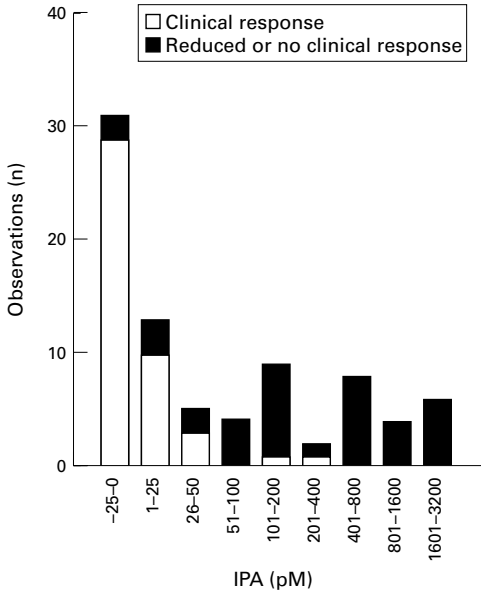

Figure 1 Frequency distribution of IPA results (pM of ^{125}I-BTX precipitated /l serum) on the initial samples from the 83 patients, divided on the basis of clinical response. Results from healthy control serum samples were subtracted from all test values.

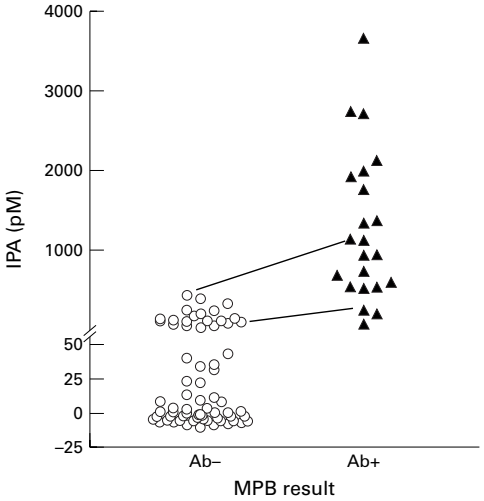

Figure 2 IPA results on the 89 samples (from 83 patients) depicted on the basis of whether the sample tested negative (open circles) or positive (shaded triangles) by MPB. The lines join the values from the two patients whose MPB Ab status changed. IPA titres ⩾50 pM are considered positive.

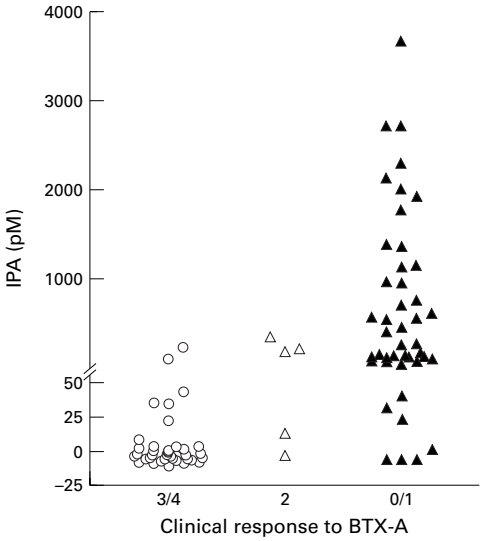

Figure 3 IPA results separated on the basis of the patient's clinical response to BTX injections. Grade 0 or 1 response indicates non-responders (shaded triangles), grade 2 response (open triangles) indicates reduced response, and grade 3 or 4 (open circles) are responders.

ued to respond to BTX-A despite low, but positive, titres (112–353). Three of these five patients had a reduced, peak effect score 2, response (fig 3).

In a previous report, we showed that lack of response to a test injection into the facial muscles is a more sensitive measure of non-responsiveness than the MPB.[18] In the present study, 29 and 19 patients respectively were given eyebrow or frontalis "test" injections, and the IPA titres corresponded well with responses to the facial "test" injections. Four patients showed no response to the eyebrow test injections despite continuing clinical response. However, three of these patients were borderline (reduced) clinical responders (peak effect score 2), who previously had a more robust response to BTX-A, and two of these patients were IPA Ab+ suggesting that the eyebrow and

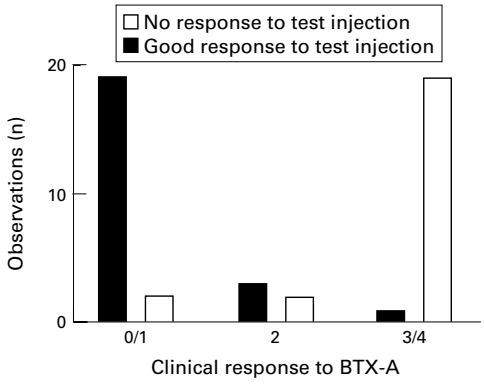

Figure 4 Correlation of clinical response (grade 0 or 1 response indicates non-responders, grade 2 response indicates reduced response, and grade 3 or 4 are responders) with response to test injections.

IPA may both be early predictors of immunoresistance.

Of the 10 clinical non-responders who also had eyebrow injections, only one had a good eyebrow response. This patient was MPB Ab− but IPA Ab+ (titre of 409 pM). Seven patients who were responders had a frontalis injection, and all seven had a good frontalis response. Of the 12 patients who were clinical non-responders and who received a frontalis injection, two had a good frontalis response. Both patients were MPB Ab− whereas one was IPA Ab+ (with a low titre, 82 pM) (see fig 4 for correlation of clinical responses with responses to "test" injections).

The specificity of both assays was relatively high, although the sensitivity of the IPA was substantially higher than the MPB (tables 1 and 2). Specificity of the MPB was 100% on all three parameters (clinical, eyebrow, and frontalis) whereas the IPA specificity was 89% for clinical ($p=0.056$, NS, Fisher's exact test), 81% for eyebrow ($p=0.226$, NS), and 89% for frontalis responses ($p=0.99$, NS). Sensitivity for the MPB was low; 50% for clinical, 38% for eyebrow and 30% for frontalis whereas the IPA sensitivity was much higher at 84% for clinical ($p<0.001$), 77% for eyebrow ($p=0.111$, NS) and 90% for frontalis responses ($p<0.02$).

The PPV of the MPB was 100% for clinical, eyebrow, and frontalis responses, whereas the NPV was 67% for clinical responses, 66% for eyebrow, and 56% for frontalis responses. The PPV of the IPA was 88% for clinical, 77% for eyebrow, and 90% for frontalis responses, whereas the NPV was 85% for clinical, 81% for eyebrow, and 89% for frontalis responses.

Sensitivity, specificity, PPV, and NPV of the individual test injections were determined in relation to clinical responses. False positives in this determination were a positive test injection response with a negative clinical response. False negatives were a negative test response with a positive clinical response. Thus, for the eyebrow injections, sensitivity was 79%, specificity was 90%, PPV was 94%, and NPV was 69%. For the frontalis injections, sensitivity was 100%, specificity was 83%, PPV was 78%, and NPV was 100%. For the test injections combined, sensitivity was 85%, specificity was 86%, PPV was 88%, and NPV was 83%.

Table 1 Clinical-immunological correlation

	Mouse bioassay (MPB)				*Immunoprecipitation assay (IPA)*			
	Ab+ (n=22)		*Ab− (n=67)*		*Ab+ (n=42)*		*Ab− (n=47)*	
Response	+	−	+	−	+	−	+	−
Clinical (n=83 subjects, 89 samples)	0	22	45	22	5	37	40	7
Eyebrow (n=29 subjects)	0	5	16	8	3	10	13	3
Frontalis (n=19 subjects)	0	3	9	7	1	9	8	1
Total responses	0	30	70	37	9	56	61	11

+=Responder; −= non-responder.

Table 2 Mouse bioassay - immunoprecipitation assay comparison

	Mouse bioassay (MPB)			*Immunoprecipitation assay (IPA)*		
Response	*Sensitivity (%)*	*Specificity (%)*	*PPV/NPV (%)*	*Sensitivity (%)*	*Specif.icity (%)*	*PPV/NPV (%)*
Clinical	50	100	100/67	84	89	88/85
Eyebrow	38	100	100/66	77	81	77/81
Frontalis	30	100	100/56	90	89	90/89

PPV=Positive predictive value; NPV=negative predictive value.

Discussion

As the number of patients treated with BTX-A continues to grow, the prevention and accurate detection of immunoresistance have become high priorities. The MPB, originally described by Hatheway and Dang,[14] has been considered by many to be the "gold standard" assay for the detection of BTX-A Ab. Here we show that an assay based on immunoprecipitation of radiolabelled BTX-A is a highly reliable test which is slightly less specific, but considerably more sensitive than the MPB. Six non-responding patients were tested twice by both assays, typically secondary to patient request or for verification purposes. Two of these were initially MPB Ab− but became Ab+ by MPB on repeat testing; the IPA values were positive on first testing and the titres rose over the 4 months between the samples (fig 2) suggesting the early detection of immunoresistance by IPA. Furthermore, there were five false positives (clinical responders with Ab+ result by IPA), but three of these patients have had declining response to BTX as well as relatively low titres by IPA, which is a quantitative test. Thus, positivity by the IPA may be a useful predictor of future non-responsiveness.

The IPA correlated well, not only with the overall clinical responses, but also with the eyebrow and frontalis "test" injections, with a specificity of 81% and 89% respectively to these upper face injections. Additionally, the strong correlation of these "test" injections with clinical response ratings provides a strong support for using these simple biological tests to evaluate patients for immunoresistance. Overall, we prefer the eyebrow injections as these are more cosmetically acceptable in that the asymmetric responses are present only during voluntary contractions whereas unilateral disappearance of frontal wrinkles may not be desirable.

The only commercially available in vitro test utilises a western blot assay. Although this test offers potential advantages over MPB in that it is less cumbersome and does not require the use of experimental animals, our previous study[18] showed that this in vitro test does not correlate as well as the MPB with clinical responses.

Based on the results or our study, we offer the following guidelines for evaluation of patients who fail to respond to BTX injections (secondary non-responders) (fig 5). When such a patient returns to the clinic after obtaining a poor or no response to the previous injection, the clinician may re-inject with the same or higher dose and/or an alteration of the site and at the same time inject 15–20 units of BTX into the right eyebrow or right frontalis. If the patient responds to either the clinical (for the primary condition—that is, dystonia) or test injection, the clinician may continue injections, possibly adjusting the dose or site of injection. If the patient shows no response to both (clinical and test) injections, the use of serological assays, such as IPA or MPB may be considered, before preceding to the next step of using other BTX serotypes,[21–23] plasma exchange, immunoadsorption, or surgery. Based on the results

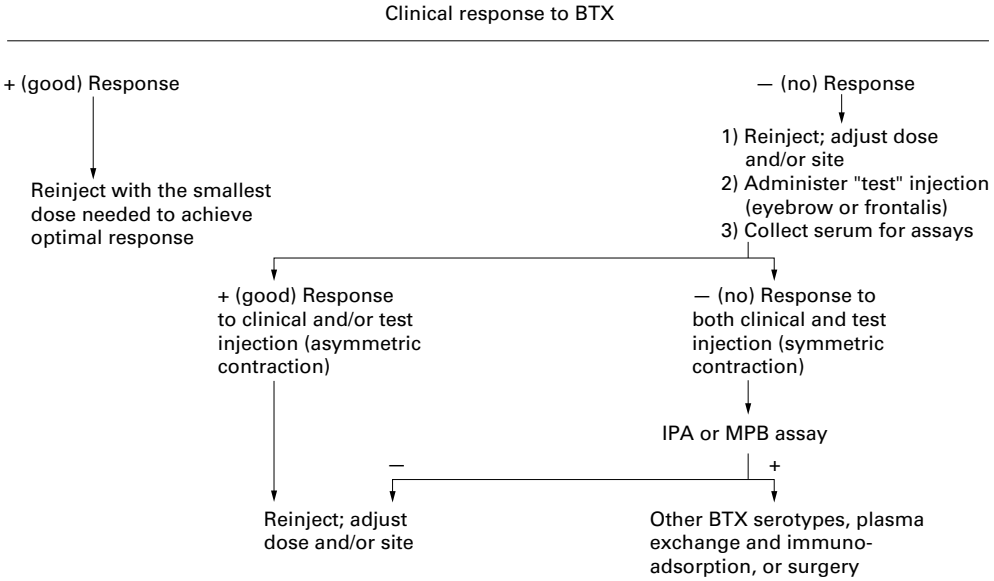

Figure 5 Decision tree for the evaluation and subsequent treatment of patients based on response to BTX injections.

of our study, we recommend the IPA assay (given the high sensitivity and specificity) as the assay of choice to confirm immunoresistance. Eight of nine patients who were clinical and test (eyebrow) non-responders were IPA Ab+, and nine of 10 patients who were clinical and frontalis non-responders were IPA Ab+. As it can be predicted with relative certainty that if both the clinical and test injections result in no response, the IPA will be positive, there may be no need to test for antibodies by the IPA in this category of patients. Given the low sensitivity of the MPB, this assay has a limited value compared with the IPA. Furthermore, the IPA does not require the use of experimental animals and it quantitatively assesses the degree of immunoresistance by providing antibody titres which can be measured serially.

It is important to recognise some possible shortcomings of our study. Although the "0–4 peak effect" scale is an established method of assessing response to BTX injections, it may not always reliably differentiate responders from non-responders. Patients were considered non-responders if they described no effect or only mild effect with no functional improvement from their most recent injection. These patients may have had suboptimal benefit from their recent injection secondary to technique, injection of inappropriate muscles, low potency of the BTX batch, or inadequate dose, and as such, the reported sensitivities of the two assays may be artificially low. A wide range of doses was given per visit at different intervals making a correlative analysis difficult. A further possible shortcoming is the definition of sensitivity and specificity used. "True positive" assumed that the Ab+ patient must be a non-responder, which is supported by our previous finding that all 20 MPB Ab+ patients had no response to BTX-A injections on at least two consecutive treatment sessions.[20] "False negatives" refer to those patients who do not respond to BTX injections despite an Ab− test.

In conclusion, our study shows that both assays have a high specificity, but because the IPA is more sensitive than the MPB and because the IPA is an in vitro assay, it may have relative advantage over the MPB. A further advantage of the IPA is that this is a quantitative assay which may be useful for serial evaluations and may have a predictive value in determining impending or future unresponsiveness. Eyebrow and frontalis "test" injections correlated well with the clinical and immunological results and can be used as reliable screening tests in patients who have either no response or an equivocal response to BTX injections.

The study was supported by grants from Allergan Pharmaceuticals and the Medical Research Council of Great Britain. We had complete control over the collection and analysis of the data.

1 Jankovic J, Brin MF. Therapeutic uses of botulinum toxin. *N Engl J Med* 1991;**324**:1186–94.
2 Jankovic J. Use of botulinum toxin in neurology. In: Kennard C, ed. *Recent advances in clinical neurology*. Vol 8. Edinburgh: Churchill Livingstone, 1995;89–110.
3 American Academy of Neurology. Assessment: the clinical usefulness of botulinum toxin-A in treating neurological disorders; report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. *Neurology* 1990;**40**:1332–6.
4 American Academy of Ophthalmology. Botulinum toxin therapy of eye muscle disorders. Safety and effectiveness. *Ophthalmology* 1989;**96**(suppl):37–41.
5 Davidson BJ, Ludlow CL. Long-term effects of botulinum toxin injections in spasmodic dysphonia. *Ann Otol Rhinol Laryngol* 1996;**105**:33–42.
6 National Institutes of Health. Consensus development conference statement on clinical use of botulinum toxin. *Arch Neurol* 1991;**48**:1294–8.
7 Clarke CE. Therapeutic potential of botulinum toxin in neurologic disorders. *Q J Med* 1992;**82**:197–205.
8 Jankovic J, Schwartz K, Donovan DT. Botulinum toxin treatment of cranial-cervical dystonia, spasmodic dysphonia, other focal dystonias and hemifacial spasm. *J Neurol Neurosurg Psychiatry* 1990;**53**:633–9.
9 Berardelli A, Formica A, Mercuri B, *et al*. Botulinum toxin treatment in patients with focal dystonia and hemifacial spasm. A multicenter study of the Italian Movement Disorder Group. *Ital J Neurol Sci* 1993;**14**:361–7.
10 Geller BD, Hallett M, Ravits J. Botulinum toxin therapy in hemifacial spasm: clinical and electrophysiological studies. *Muscle Nerve* 1989;**12**:716–22.
11 Scott AB. Foreword. In: Jankovic J, Hallett M, eds. *Therapy with botulinum toxin*. New York: Marcel Dekker, 1994:vii–viii.
12 Zuber M, Sebald M, Bathien N, *et al*. Botulinum antibodies in dystonic patients treated with type A botulinum toxin: frequency and significance. *Neurology* 1993;**43**:1715–18.
13 Siatkowski RM, Tyutyunikov A, Biglan AW, *et al*. Serum antibody production to botulinum A toxin. *Ophthalmology* 1993;**100**:1861–6.
14 Hatheway CH, Dang C. Immunogenicity of the neurotoxins of Clostridium botulinum. In: Jankovic J, Hallett M, eds. *Therapy with botulinum toxin*. New York: Marcel Dekker, 1994:93–107.
15 Notermans S, Dufrenne J, Van Schothorst M. Enzyme-linked immunosorbent assay for detection of Clostridium botulinum toxin type A. *Japan J Med Sci Biol* 1978;**31**:81–5.
16 Notermans S, Nagel J. Assays for botulinum and tetanus toxins. In: Simpson LL, eds *Botulinum neurotoxin and tetanus toxin.* San Diego: Academic Press, 1989;319–331.
17 Shone C, Wilton-Smith P, Appleton N, *et al*. Monoclonal antibody-based immunoassay for type A Clostridium botulinum toxin is comparable to the mouse bioassay. *Appl Environ Microbiol* 1985;**50**:63–7.
18 Hanna PA, Jankovic J. Mouse bioassay versus western blot assay for botulinum toxin antibodies: correlation with clinical response. *Neurology* 1998;**50**:1624–9.
19 Palace J, Nairne A, Hyman N, *et al*. A radioimmunoprecipitation assay for antibodies to botulinum A. *Neurology* 1998; **50**:1463–6.
20 Jankovic J, Schwartz, KS. Response and immunoresistance to botulinum toxin injections. *Neurology* 1995;**45**:1743–6.
21 Eleopra R, Tugnoli V, Rossetto O, *et al*. Botulinum neurotoxin serotype C: a novel effective botulinum toxin therapy in human. *Neurosci Lett* 1997;**224**:91–4.
22 Houser MK, Sheean GL, Lees AJ. Further studies using higher doses of botulinum toxin type F for torticollis resistant to botulinum toxin type A. *J Neurol Neurosurg Psychiatry* 1998;**64**:577–80.
23 Lew MF, Adomato BT, Duanne DD, *et al*. Botulinum toxin B (BotB): a double-blind, placebo-controlled, safety and efficacy study in cervical dystonia. *Neurology* 1997;**49**:701–7.