Determination of Anti-Acetylcholine Receptor Antibodies in Myasthenic Patients by Use of Time-resolved Fluorescence

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Background: Autoantibodies against nicotinic acetylcholine receptor (nAChR) in myasthenia gravis (MG) patients are usually detected by radioimmunoprecipitation assays using extracted acetylcholine receptors labeled irreversibly with 125I-α-bungarotoxin (α-BuTx). To provide a nonradioactive immunoassay, we established an assay using nAChRs labeled with Eu3+-α-cobratoxin (α-CTx). We derivatized α-CTx with a diethylenetriaminepentaacetic moiety and formed a complex with Eu3+. The complex was purified by HPLC, and the fractions were tested for binding to Torpedo nAChRs and human nAChRs. The most active fractions were used to label nAChRs for the immunoprecipitation assay, and the bound Eu3+ was quantified by time-resolved fluorescence.

Results: Eu3+-labeled α-CTx competed with 125I-α-BuTx for binding to Torpedo nAChRs and saturated the binding sites of human nAChRs, with a Kd of 7.2 × 10^-9 mol/L. Results of the immunoassay performed with Eu3+-labeled α-CTx were similar to those obtained with 125I-α-BuTx, with a slightly higher limit of detection [0.3 nmol/L (n = 6) vs –0.1 nmol/L for isotopic assay]. None of 34 negative sera tested (16 healthy controls, 10 patients with nonmyasthenia-related disease, 8 patients seronegative for MG) gave a value >0.3 nmol/L. Of the 35 positive myasthenic sera (with antibody values, previously determined by isotopic assay, of 0.4–1290 nmol/L) compared in the two assays, 32 tested positive with the Eu3+ assay. Linear regression analysis yielded the equation: y = 1.035x − 0.013 nmol/L; Syx = 0.172 nmol/L; r² = 0.977.

Conclusions: The new time-resolved fluorescence method for quantification of antibodies to nAChRs in MG patients provides a performance similar to that of the widely used isotopic assay and could be used in laboratories with restricted use of isotopes.

Myasthenia gravis (MG) is a human autoimmune disorder manifested by muscle weakness and fatigability, occurring with a prevalence of 10–100 per 1 000 000 (1). The antigenic target is the nicotinic acetylcholine receptor (nAChR) of skeletal muscles. Detection and quantification of anti-nAChR antibodies in the serum of patients is used for the diagnosis of the disease and monitoring its course. Assays are mostly based on the design introduced by Lindstrom et al. (2) and use the immunoprecipitation of solubilized nAChRs prelabeled with 125I-α-bungarotoxin (125I-α-BuTx). The procedure is widely used, specific, sensitive, and thoroughly validated. However, working with radioactivity is associated with problems, such as limited life span of the labeled reagent, potential health hazards, and complicated disposal of radioactive waste.

High-sensitivity detection of lanthanides (comparable to the sensitivity for detection of 125I) is possible by the use of time-resolved fluorescence methods, and those labels are used for measurement of numerous compounds (3–6). However, lanthanides cannot be used for direct labeling, and the compound to be detected (e.g., a protein) must first be derivatized with a suitable chelate, which is then complexed with the lanthanide.

We describe the preparation of Eu3+-labeled α-cobratoxin (α-CTx; long neurotoxin from Naja naja kaouthia, nonstandard abbreviations: MG, myasthenia gravis; nAChR, nicotinic acetylcholine receptor; α-BuTx, α-bungarotoxin; α-CTx, α-cobratoxin; LOD, limit of detection; and DTPA, diethylenetriaminepentaacetic acid.)
which is highly similar to α-BuTx), which can be measured similar to the so-called dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA), and demonstrate its use in an immunoassay for antibodies to nAChR. The assay procedure is analogous to that in routine use (2) and shows identical clinical specificity and an only slightly higher limit of detection (LOD).

**Materials and Methods**

**Material and Reagents**

Venoms of *Bungarus multicinctus* and *N. naja kaouthia* were obtained from Latoxan; 125I-labeled α-BuTx (specific activity, 8.2 TBq/mmol or 74 TBq/mmol) was from Amersham Pharmacia Biotech; diethylenetriaminepentaacetic acid (DTPA) anhydride and Eu(NO3)3 were from Sigma-Aldrich; Sephadex G-25, CM-Sephadex C-50, and mono-Q 5 × 5 columns were from Pharmacia Biotech; octylSi300 (5 μm) HPLC support was from Serva; antihuman IgG (QSwAHulG) was from Sevapharma; DELFIA enhancement solution was from Wallac Oy; TE671 cell culture was from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; and *Torpedo marmorata* electric organs were from the Station Biologique d’Arcachon. All other chemicals were of analytical grade and were purchased mostly from Sigma-Aldrich.

**Purification of Toxins**

α-CTx was purified from the venom of *N. naja kaouthia* by the procedure of Karlsson et al. (7). α-BuTx was purified from the venom of *B. multicinctus* by a procedure similar to that described by Lee et al. (8). Both toxins showed a single peak when tested by various HPLC analyses and were estimated to be at least 96% pure.

**CTx Labeling and Purification of Derivatives**

To 2 mg (0.25 μmol) of α-CTx in 0.6 mL of 150 mmol/L sodium carbonate (pH 9.3), we added 16 mg of DTPA anhydride and Eu(NO3)3 and further purified by reversed-phase and ion-exchange chromatography. The Eu3+-treated fraction was loaded onto a 8 × 80 mm column packed with octylSi300 (Serva) and eluted at 1 mL/min with the following gradient: 10% B for 5 min; 10–70% B over 120 min; 70% B for 15 min; 70–10% B over 10 min. The gradient system was as follows: solvent A, 1 g/L trifluoroacetic acid in water; solvent B, 1 g/L trifluoroacetic acid in 700 mL/L acetonitrile. The absorbance at 280 nm was monitored, and 4-mL fractions were collected.

Fractions from reversed-phase chromatography were further purified on a mono-Q column (5 × 50 mm; Pharmacia) eluted at 0.5 mL/min with the following gradient profile: 0% B for 10 min; 0–40% B over 90 min; 40–100% B over 20 min. The gradient system was as follows: solvent A, 20 mmol/L ammonium acetate in 100 mL/L methanol; solvent B, 500 mmol/L ammonium acetate in 100 mL/L methanol. The absorbance at 280 nm was monitored, and 2-mL fractions were collected and concentrated by lyophilization.

**Determination of the Affinity of Derivatized α-CTx for Nicotinic Receptors**

The relative affinity was determined by competition experiments in which different amounts of the derivatized α-CTx inhibited the binding of 125I-α-BuTx to AChR prepared from the electric organ of *T. marmorata*, according to the method of Fels et al. (9).

**Culture of TE671 Cells**

The rhabdomyosarcoma cell line of human origin, TE671, was the source of the nAChR. The use of this cell line was introduced for measurements of antibodies in myasthenia (10–12). Cells were grown, essentially as described by Somnier (13), in DMEM containing 2 mmol/L glutamine and 100 mL/L fetal bovine serum in glass bottles (~180 cm2) in 5% CO2 atmosphere at 37°C, with seeding of ~3 × 106 cells/bottle. On the 4th day, when cells approached confluence, the fetal bovine serum concentration was reduced to 10 mL/L, and the medium was supplemented with 2.5 μmol/L dexamethasone and 100 μmol/L nicotine. Cells were harvested on the 7th day of culture, washed, and centrifuged, and the pellet was kept at –65°C until further processing.

**Preparation and Labeling of Human Nicotinic Receptors**

Triton X-100 extracts containing the receptors were prepared from human muscles (mostly from amputated legs from patients with complicated diabetes) by the procedure described by Lindstrom et al. (14) or from TE671 cells as described by Voltz et al. (15). nAChR complexed with Eu3+-labeled α-CTx was prepared so that ~0.5 nmol/L nAChR was saturated to 60–75% with the labeled toxin. The concentration required was first established by binding studies using different concentrations of the Eu3+-labeled α-CTx.

**Isotopic Assay of Myasthenic Antibodies**

The procedure described by Lindstrom et al. (14) was used, with volumes scaled down fivefold. The detection
limit of the assay was \( \sim 0.1 \text{ nmol/L} \). The final reaction volume of 0.2 mL contained 0.2 nmol/L nAChR, 0.3 nmol/L \( ^{125}\text{I}\)-\( \alpha\)-BuTx, and 2–5 \( \mu \text{L} \) of the investigated human serum. If required for quantitative determination of antibody, the sera were first diluted with a negative control serum.

**Eu\(^{3+}\) Assay**

We incubated 0.2 mL of nAChR-Eu\(^{3+}\)-\( \alpha\)-CTx complex (nAChR concentration \( \sim 0.5 \text{ nmol/L} \)) with 10 \( \mu \text{L} \) of human serum for 2 h at room temperature and then overnight at 4 °C. Human IgG was then precipitated with anti-human IgG (100 \( \mu \text{L} \) of Q5wAHulG) during a 5-h incubation at room temperature. The resulting precipitate was centrifuged (10 min at 10 000 g and 4 °C), washed four times with 1 mL of phosphate-buffered saline, centrifuged (10 min at 10 000 g and 4 °C), and dissolved in 0.1 mL of “enhancement solution” (100 mmol/L acetate-phthalate, pH 3.2, 15 \( \mu \text{mol/L} \) 2-naphthoyltrifluoroacetone, 50 \( \mu \text{mol/L} \) tri-\( \pi \)-octylphosphine oxide, 1 mL/L Triton X-100). After an overnight incubation at room temperature, the contents were transferred from the test tubes to a microtitration plate, and time-delayed fluorescence was measured with a Victor 1420 Multilabel Counter (Wallac Oy), using the factory-set mode for Eu\(^{3+}\).

**Sera**

Sera from 43 MG patients (14 males, 29 females; age range, 22–85 years) with titers covering a wide range were used in this study; 8 of those patients were seronegative by the isotopic assay. The severity of their disease ranged from group I to group III as graded according to the scale proposed by Osserman (16). Diagnosis of MG was based on typical clinical, pharmacologic (17), electromyographic (18), immunologic, and neurophysiologic features, and the radiologic status of the thymus was checked by computerized tomography of the mediastinum. The Neurologic Clinic registered 1185 MG patients from the Czech Republic (457 males and 728 females). Sera from healthy blood donors (n = 16) and patients with nonmyasthenic diseases (5 with multiple sclerosis, 5 with lupus erythematosus) served as negative controls. Laboratory workers worked with numbered samples without knowledge of the diagnosis.

**Results**

**Preparation of the Eu\(^{3+}\)-Labeled \( \alpha\)-CTx Derivative**

Approximately 60% of \( \alpha\)-CTx was derivatized by DTPA under the described conditions. The Eu\(^{3+}\)-labeled toxin was analyzed by reversed-phase HPLC, and several positional isomers were detected (Fig. 1). Fractions isolated by HPLC were assayed for their relative affinity for nAChR by measuring their ability to inhibit the binding of \( ^{125}\text{I}\)-\( \alpha\)-BuTx to nAChR from electric organ membrane fragments. Fig. 2 shows the inhibition of binding of \( ^{125}\text{I}\)-\( \alpha\)-BuTx to AChRs by native and Eu\(^{3+}\)-labeled \( \alpha\)-CTx.

The concentration of Eu\(^{3+}\)-labeled \( \alpha\)-CTx required for half-maximal saturation of nAChR was \( \sim 50 \) times higher than the respective concentration of the native \( \alpha\)-CTx.

Derivatives eluted at 0–60 mL showed no measurable affinity for nAChR and contained \( \sim 30% \) of the applied toxin. Fractions eluting at \( \sim 110, 150, \) and 190 mL showed binding to nAChR, but their contribution was minor both in protein content and total binding capacity. The peak eluting between 60 and 90 mL contained \( \sim 60\% \) of the derivatized \( \alpha\)-CTx and the bulk of the total binding capacity for nAChR. Analysis by ion-exchange HPLC on a mono-Q column showed that this fraction contained three positional isomers, all of which were actively bound to nAChR. Therefore, a pool of the fractions eluting between 60 and 90 mL was used in the present work.
BINDING OF EU³⁺-LABELED α-CTX TO RECEPTORS

To measure the binding of EU³⁺-labeled toxin to solubilized nAChRs from human muscle, we used immunoprecipitation with a highly positive MG serum. Binding of our EU³⁺-labeled toxin preparation was specific and saturable (Fig. 3A), with two different apparent affinities: 3.7 × 10⁻⁷ mol/L (−40%) and 145 × 10⁻⁹ mol/L (−60%). For comparison, similar results with ¹²⁵I-α-BuTx are shown in Fig. 3B.

TITRATION OF MYASTHENIC SERA IN EU³⁺ ASSAY

To detect antibodies in myasthenic sera, we used 100 fmol of nAChR saturated to 60–75% with EU³⁺-α-CTX. The EU³⁺-α-CTX-nAChR complexes were prepared “in bulk” and were stable for at least 1 year if stored in appropriate aliquots (to avoid repeated thawing and freezing) at −80 °C (data not shown). Receptors extracted from both sources (TE 671 cells or human muscles) showed similar suitability for the assay.

Examples of the titration of individual myasthenic sera are shown in Fig. 4. Taking into account the differences in specific activity of the two toxins, the shapes of individual titration curves obtained were very similar. The specific activity of the EU³⁺-labeled α-CTX preparation used in this study was 1500 fluorescence counts/mmol. Negative sera typically gave 11300 ± 1050 (mean ± SD) fluorescence counts, and the same background values were obtained when specific binding of the label was blocked by an excess of cold α-BuTx (10⁻⁷ mol/L).

We used three 10-μL serum aliquots for the initial estimation of the titer. If >20% of the available EU³⁺-α-CTX-nAChR complex precipitated, the serum was diluted with negative control serum (to maintain the immunoglobulin concentration) so that at least two dilutions were in the range of 30% of the precipitated complex. The titration data were plotted and fitted with a hyperbolic curve, and the resulting titer was calculated from the initial asymptote.

LOD, PRECISION, AND DIAGNOSTIC ACCURACY OF EU³⁺ ASSAY

We made an effort to adhere to the published guidelines for diagnostic accuracy for our test (19). The calculated LOD, determined as 3 SD of the blank with 10 μL of serum and EU³⁺-α-CTX-nAChR complex at 70% saturation, was 0.3 nmol/L. None of the negative sera tested gave a value exceeding that limit. The typical “within-run” CV for triplicates in the isotopic assay was ~5%. The imprecision of the fluorescence method was greater, with a within-run CV, for triplicates, of ~10% and a between-run CV ~16%. The usefulness of the fluorescence method, however, was demonstrated by comparison of titers obtained by the two methods (Fig. 5). We compared 43 myasthenic sera (with titer values between 0.3 and 1290 nmol/L as determined by the isotopic assay); 8 of those sera (not included in Fig. 5) were seronegative by the isotopic assay, with titers indistinguishable from those of healthy controls (titer ≤0.3 nmol/L), and were negative by the EU³⁺ assay as well. We included eight sera with titers of 0.4–1 nmol/L; three of those gave values ≤0.3 nmol/L in the EU³⁺ assay. Two of these three were also near the LOD for the isotopic assay; 0.4 nmol/L. The third serum that was negative in the EU³⁺ assay gave a titer of 0.7 nmol/L in the isotopic assay. Overall, the linear regression of data yielded the following equation: y = 1.035x − 0.013 nmol/L (Sₓᵧ = 0.172 nmol/L; r² = 0.977).

Discussion

We established a nonradioactive immunoassay for antibodies to AChRs that used EU³⁺-labeled α-CTX rather than ¹²⁵I-α-BuTx. The reagent used to label the antigen is more stable than ¹²⁵I-α-BuTx, the concentration of antibodies can be expressed in molar units as in conventional RIAs, and the results show similar detection limits and identical diagnostic specificity.
Several nonradioactive methods for the detection of anti-nAChR antibodies have been described previously. One approach is based on solid-phase attachment of nAChR and detection of bound anti-nAChR immunoglobulin by labeled anti-immunoglobulin antibody (20–23); another is on the substitution of horseradish peroxidase-labeled $^{125}$I-BuTx for $^{125}$I-BuTx in the immunoassay (24). None of these methods has been adopted for routine use, probably because they are less sensitive than the $^{125}$I-BuTx radioimmunoprecipitation assay.

We attempted to use Eu$^{3+}$-labeled $\alpha$-BuTx, but the derivatives of $\alpha$-BuTx that we obtained lost much of their affinity for nAChRs. Labeling of $\alpha$-CTx produced a useful derivative. Our preparation probably represents a mono-substituted toxin with DTPA conjugated with either lysine or the N-terminal amino acid (proline). It would be possible to isolate individual positional isomers, but our procedure has been worked out for a mixture of several isomers that are isolated together. Binding analysis of the HPLC-purified fraction used for the experiments suggested the presence of two main components with calculated binding affinities of $3.7 \times 10^{-9}$ mol/L and $145 \times 10^{-9}$ mol/L.

As was already noted, the results obtained with the Eu$^{3+}$ assay correlate closely to the results obtained with the isotopic assay. This was to be expected because the design of both methods is almost identical and the only important difference is in the label used for the $\alpha$-toxin. The described fluorescence assay can measure concentrations of anti-nAChR antibodies $>0.3$ nmol/L, but reliable estimates can be expected for titers $>0.5$ nmol/L. This LOD is slightly higher than that of the isotopic assay, which has a LOD in the range of 0.1–0.2 nmol/L. In MG, titers $>0.5$ nmol/L are considered positive, whereas titers of 0.2–0.5 nmol/L are evaluated as equivocal [e.g., see Vincent and Newsom-Davis (25)]. Although the isotopic assay might be more advantageous for such "equivocal" cases, the advantages of the nonradioactive fluorescence assay that we describe may make it a useful tool in the diagnosis and follow-up of myasthenic patients.

Fig. 4. Titration of myasthenic sera using $^{125}$I-$\alpha$-BuTx (left panels) and Eu$^{3+}$-$\alpha$-CTx-nAChR complex (right panels).

Values for three sera (low (1.1 nmol/L), intermediate (42 nmol/L), and high (1290 nmol/L) titer as determined by the isotopic assay) are shown. Data are fitted with hyperbolic function, and the slope calculated from the fit is used for the titer quantification.
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