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Antibodies to motor endplates demonstrated with the immunofluorescence technique

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SUMMARY

Antibodies to acetylcholine receptors (AChR) are probably directly responsible for the pathogenesis of myasthenia gravis (MG). Methods for the demonstration of these antibodies are complicated. The present study shows that the antibodies can also be revealed by the use of the simple indirect immunofluorescence technique with rat diaphragm as substrate. Antibodies were demonstrated with FITC-labelled anti-human Ig. The location on the motor endplates was confirmed by using a TRITC-labelled anti-α-bungarotoxin system. Antibodies to motor endplates were only demonstrated in MG and not in either twenty-two patients with neuromuscular disorders or fifty normal subjects. Antibodies to motor endplates were found in only twelve out of fifty-seven MG patients. In fifteen of the other forty-five patients, antibodies were found of the classical anti-skeletal muscle type, 'overluminating' the anti-motor endplate antibodies.

INTRODUCTION

In 1973 it became clear that auto-antibodies to acetylcholine receptors (AChR) might be involved in myasthenic syndromes (Patrick & Lindström, 1973). Since then, Almon, Andrew & Appel, (1974) and Appel, Almon & Levy, (1975) using radio-immunological methods, Bender *et al.* (1975) using an immunohistological method and Aharonov *et al.* (1975) using a complement fixation technique, have succeeded in demonstrating antibodies to AChR in about 75% of sera from patients with myasthenia gravis (MG). The first two methods used α-bungarotoxin (α-BT) as an indicator of AChR.

None of the above methods is suited to test great numbers of sera. Even the more advanced radio-immunological methods of Lindström (1977) and Monnier & Fulpius (1977), who also used α-BT as marker for AChR, are hampered because muscle tissue from human leg amputations is used to prepare the AChR substrate. The muscle tissue of one leg is generally only sufficient for about fifty tests.

Therefore, we wanted to study whether the indirect immunofluorescence technique could be used for the demonstration of anti-AChR antibodies, by performing a test using fluorescent antibodies to α-BT. If, as Bender *et al.* (1975) showed, antibodies to AChR inhibit a subsequent binding of α-BT, this would lead to a diminished fluorescence after the application of the anti-α-BT conjugate.

We were pleasantly surprised to note that, with a simple indirect immunofluorescence technique using a fluorescent anti-human Ig conjugate, antibodies to motor endplates could be demonstrated. α-BT and the anti-α-BT conjugate were now only used to confirm the motor endplate location. As it is not proven that the antibodies determined in this way are directed to AChR, we prefer, for the time being, to report on antibodies to motor endplates.

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MATERIALS AND METHODS

Patients. Sera from fifty-seven MG patients, twenty-two patients with various neuromuscular disorders (Table 1) and fifty healthy blood donors were stored at −20°C. The diagnosis of MG was confirmed by one of us (H. K. van Walbeek) or by H. J. G. H. Oosterhuis, Neurological Department, University Hospital, Groningen. The diagnosis was based on the presence of a fluctuating muscular weakness, which could be beneficially influenced by rest and cholinesterase inhibitors and worsened upon activity of the muscle concerned. A prerequisite for the diagnosis was that the weakness involved at least one group of muscles innervated by a cranial nerve.

TABLE 1. Diagnosis of twenty-two patients with various neuromuscular disorders

Disorder	Number
Peroneal muscular atrophy	1
Benign infantile spinal muscular atrophy	3
Amyatrophyc lateral sclerosis	1
Myotonia congenita	1
Nemaline myopathy	3
Duchenne's disease	3
Polymyoistis	1
Facio scapulo humoral muscular dystrophy	2
Primary muscular dystrophy, unknown origin	4
Primary neural disorder, unknown origin	3

α-Bungarotoxin (α-BT). The purified toxin (batch BM α 51-1) was obtained from Miami Serpentina Laboratories. A stock solution of 1·0 mg/ml phosphate buffered saline (PBS) was stored at −20°C.

Rabbit anti-α-BT. Rabbits were immunized with 0·1 mg α-BT once a month for 10 months. For the first immunization, the antigen was mixed with its own volume of Freund's complete adjuvant. The subsequent eight immunizations were performed with the same amount of antigen with Freund's incomplete adjuvant. The antiserum (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, lot no. 0976) was tested for purity with a double immunodiffusion method.

Conjugates. Horse anti-human Ig conjugated to fluorescein isothiocyanate (FITC), (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, lot no. PH 17-4-F8, prot. conc. 14·5 mg/ml, mol F/P ratio 2·5, final dilution 1/80) was used for the demonstration of antibodies to motor endplates. Horse anti-rabbit Ig conjugated to tetramethylrhodamine isothiocyanate (TRITC) (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, lot no. PK 17-2-T2, prot. conc. 4·1 mg/ml, mol T/P ratio 2·7, final dilution 1/70) was used for the location of motor endplates.

Immunofluorescence technique. The indirect immunofluorescence technique on rat diaphragm was performed as described previously (Feltkamp & Van Rossum, 1968; Feltkamp, 1975). The only modification was that, in the present study, sections of 4·0 μm instead of 2·0 μm thickness were used. The serum to be studied was diluted 1/10; all washings were for 30 min at 22°C. For the excitation of TRITC, we used an HBO 150-W mercury lamp, the excitation filters KP 560, BG 36 and OG 515, a dichroic mirror TK 580 and a barrier filter 580. For the excitation of FITC, we used an XBO 75-W xenon lamp and as excitation filters, two KP 490, yellow filter 445, a dichroic mirror TK 510 and a barrier filter 515.

Cholinesterase staining. The method according to Karnovsky & Roots (1964) was used.

RESULTS

Unfixed rat diaphragm was a satisfactory substrate for the determination of antibodies to motor endplates. Sections were successively incubated with one drop of an α-BT solution, containing 0·125 μg/ml, with one drop of a 1/20 dilution of the rabbit anti-α-BT serum and one drop of the TRITC-conjugated horse anti-rabbit-Ig conjugate. The fluorescence pattern might well have represented motor endplates. No fluorescence was seen when PBS was used instead of α-BT, or normal rabbit serum instead of the anti-α-BT antiserum.

For further confirmation, serial sections were stained for cholinesterase according to Karnovsky & Roots (1964) (Fig. 1). It became clear that the α-BT binding sites also showed this enzyme. Therefore,

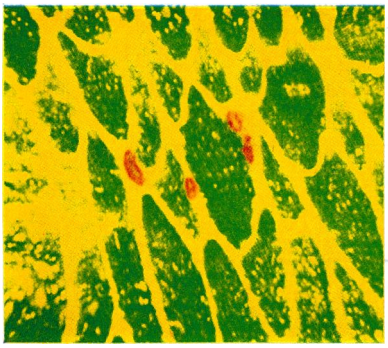

FIG 1.

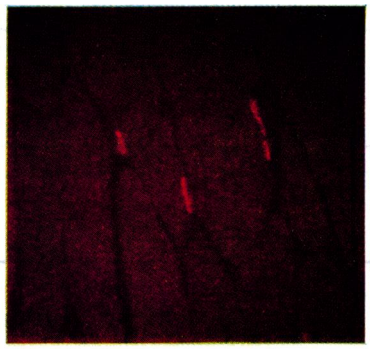

FIG 2a.

FIG 2b.

FIG. 1. Cryostat section of rat diaphragm stained for cholinesterase according to Karnovsky & Roots (1964).

FIG. 2. Cryostat section (serial section from Fig. 1) successively incubated with: (1) serum from a patient with MG, (2) FITC-labelled anti-rabbit Ig conjugate, (3) α-BT, (4) rabbit anti-α-BT antiserum, and (5) TRITC-labelled anti-rabbit Ig conjugate. Photo (a) with KP 560, BG 36, OG 515 excitation filters, TK 580 dichroic mirror and 580 barrier filter. Photo (b) with two KP 490, yellow filter 445 excitation filters, TK 510 dichroic mirror and 515 barrier filter. (a) Represents the location of the endplates by α-BT and (b) the anti-motor endplate antibodies.

we concluded that these structures represented motor endplates. Each section of rat diaphragm contained about twenty-five of these endplates.

To see whether antibodies to AChR could be demonstrated by the inhibition of a subsequent binding of α-BT, sera from fifty-seven MG patients were studied at a dilution of 1/10. Clear inhibition observed in none. It is probable therefore, that the determinant for the anti-AChR antibodies and the binding site of α-BT are not identical.

It might have been possible that none of these sera contained anti-AChR antibodies. To see whether the same pattern, as shown in Figs 1 and 2a, could be observed we utilized the classical indirect immunofluorescence technique. In a few sera, the classical pattern of alternating striations, probably indicating antibodies to sarcoplasmic reticulum (Mendell, Whitaker & Engel, 1973), was observed. With some of the other sera, however, we saw patterns identical with those obtained with α-BT and with the cholinesterase staining.

The identity of these images was proved by performing double-staining experiments. For this, the sections were successively incubated with: (1) the patient's serum, (2) FITC-labelled anti-human Ig conjugate, (3) α-BT, (4) rabbit anti-α-BT antiserum, and (5) TRITC-labelled anti-rabbit Ig conjugate. With a microscope equipped for double staining, it became clear that the location of the green (FITC) fluorescence (Fig. 2b) and the red (TRITC) fluorescence (Fig. 2a) was exactly the same. This does not exclude the fact that, on the molecular level, the determinants for the antibodies and the binding site for α-BT are different.

In sera with antibodies against the sarcoplasmic reticulum of skeletal muscle, the overall fluorescence of the alternating striations 'overluminates' the delicate fluorescence of the motor endplates. This was demonstrated by mixing sera with antibodies to sarcoplasmic reticulum with sera containing antibodies to motor endplates.

In fifteen of the fifty-seven sera from MG patients, antibodies to sarcoplasmic reticulum were present (Table 2). In twelve of the other forty-two sera, antibodies to motor endplates were found. The titre of the antibodies was determined from five sera. Titres between 1/40 and 1/160 were found. In one serum, in which only antibodies to sarcoplasmic reticulum were observed at the 1/10 dilution, a dilution series revealed the presence of antibodies to motor endplates up to a dilution of 1/320. The anti-sarcoplasmic reticulum antibodies were negative at that dilution.

None of the twenty-two patients with various neuromuscular disorders (Table 1), diagnosed after

TABLE 2. Frequency of antibodies to skeletal muscle and motor endplates

	Number studied	Antibodies to SR* (%)	Antibodies to ME† (%)	Total (%)
MG patients	57	26	21	47
Neuromuscular disorders	22	0	0	0
Normal subjects	50	0	0	0

* Sarcoplasmic reticulum.
† Motor endplates.

TABLE 3. Relation between the severity of MG and antibodies to sarcoplasmic reticulum (SR) and motor endplates (ME)

Clinical state	Number studied	anti-ME	anti-SR	Total
Ocular MG	3	0	0	0
Mild, generalized MG	20	4	4	8
Moderate, generalized MG	22	6	4	10
Severe (respiratory impairment) MG	6	1	4	5
Remission	6	1	3	4

routine clinical, biochemical and electromyographic examination, had antibodies to motor endplates. All sera from normal subjects gave negative results.

From Table 3 it can be seen that the presence of antibodies to motor endplates or sarcoplasmic reticulum was related to the severity of the disease (correlation coefficient, $r = 0{\cdot}31$), the patients in remission not being considered.

DISCUSSION

The methods used for the determination of antibodies to AChR are laborious and complicated. The method described in the present study is, on the contrary, just a simple indirect immunofluorescence technique, which is familiar in most laboratories equipped to study auto-immune diseases. The only addition that is necessary to locate the indirect fluorescence of the auto-antibodies, is a further incubation with α-BT and anti-α-BT labelled with another fluorochrome.

It is astonishing that antibodies to motor endplates have not been discovered before. We, ourselves, must have seen them for 15 years without realizing their presence. This is explained by the fact that antibodies to skeletal muscle sarcoplasmic reticulum antigens impress so much that the tiny streaks, representing antibodies to motor endplates, are easily overlooked.

The great drawback of the present method is the low sensitivity. Only twenty-seven out of fifty-seven MG patients were positive (47% had either anti-sarcoplasmic reticulum antibodies and/or anti-motor endplate antibodies). Lindström (1977) and Monnier & Fulpius (1977) found, with their radioimmunoprecipitation method, antibodies to AChR in over 90% of MG patients. Exchange of thirty-one sera from MG patients and controls between Dr B. W. Fulpius and our laboratory revealed a reasonable agreement.

The high sensitivity obtained with the radioimmunoprecipitation method could only be reached by the use of AChR of human instead of animal origin. In the present study we used rat diaphragm. Future studies will show whether fresh muscle tissue of human origin is also a better substrate for the immunofluorescence technique.

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