

Acta Medica Okayama

Volume 29, Issue 6

1975

Article 1

DECEMBER 1975

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Abstract

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Acta Med. Okayama 29, 397—404 (1975)

**AUTOANTIBODIES IN MYASTHENIA GRAVIS:
DEMONSTRATION OF ANTI-MOTOR ENDPLATE
ANTIBODY AND ANTI-MUSCLE MEMBRANE
ANTIBODY USING MEMBRANE IMMUNO-
FLUORESCENCE TECHNIQUE**

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Received for publication, May 20, 1975

Abstract: The presence of specific serum antibodies in five myasthenia gravis patients was demonstrated against the motor endplates and muscle membranes of rats by membrane immunofluorescence technique. The immunologic specificity of the antibodies was confirmed. The clinical significance is discussed.

Our previous studies on myasthenia gravis (MG) have demonstrated the presence of serum antibodies against cross-striations (1) and muscle membrane (2) of rat skeletal muscles. In the present paper, we report on antibodies against the motor endplates and muscle membranes of rat skeletal muscle using membrane immunofluorescence technique (3).

MATERIALS AND METHODS

Samples of muscle membrane were isolated from the intercostal muscles of rats by the method described previously (4). The sites of the motor endplates in the isolated muscle membrane were confirmed using methylene blue (5) or cholinesterase staining (6).

Five MG patients were selected according to Osserman's classification (7). There were two type IIa cases and one case each of type IIb, III and IV. Three of these five patients had been thymectomized. Twenty ml of blood was collected from each patient, and the serum separated. Gamma-G immunoglobulin (IgG) fraction was purified from the serum by fractionation with saturated ammonium sulfate followed by DEAE-cellulose column chromatography, and the product was sufficiently absorbed with washed rat erythrocytes. Fluorescein isothiocyanate (FITC) was then conjugated with purified IgG (F : P = 4 : 1), and the excess was eliminated by passing the labelled immunoglobulin fraction through a column of Sephadex G-25 to obtain the FITC-conjugated IgG. The control material was FITC-conjugated IgG prepared from a healthy subject by the same procedures. These FITC-conjugated IgG preparations were absorbed with isolated muscle membranes for one hour at 37°C or 4°C to obtain absorbed

FITC-conjugated IgG.

To locate and identify specific antibodies against the muscle membrane and motor endplate, the isolated muscle membrane and patient serum were incubated together in test tubes at 37°C, 18°C or 4°C, for graded lengths of time (15, 30, 60 or 120 min or 12 hr). The mixtures were washed by centrifugation with four changes of 0.1 M phosphate buffered saline (PBS), pH 7.2, and allowed to react with the FITC-conjugated antihuman IgG for 30 min at 18°C. The mixtures were again washed with PBS four to ten times and the resultant deposits were mounted on glass slides and sealed with glycerol-PBS.

In the direct immunofluorescence test, the FITC-conjugated IgG was placed on the isolated muscle membrane, which was allowed to stand at 37°C, 18°C or 4°C for 30 min and then washed with PBS ten times.

These slides were examined with a fluoromicroscope (NIKON), using BO and No. 1 medium filters with BV excitation. Photomicrographs were taken with Kodak Tri-X film exposed for 2 to 5 min and developed with Pandol^R at 20°C for 14 min in a hypersensitizing tank.

RESULTS

In indirect membrane immunofluorescence, the entire muscle membrane was stained green with the motor endplate of the membrane appearing as a more deeply stained green structure. Green-stained terminal innervations of the axon were occasionally observed (Fig. 1). Cross-striations of myofibrils in

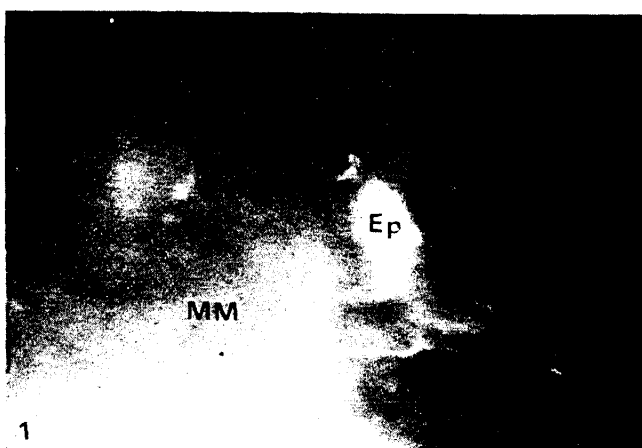


Fig. 1. Indirect membrane immunofluorescence. The serum of the MG patient was sandwiched when the preparation was stained with FITC-conjugated antihuman IgG. The entire muscle membrane (MM) was stained green, with the motor endplate situated on the membrane appearing as a deep green structure (Ep). Terminal innervation of the axon (Ti) was stained green. $\times 400$.

conjunction with muscle membrane were found stained in the same manner (Fig. 2). In control serum, the muscle membrane was stained yellowish-brown and the motor endplate, yellowish (Fig. 3). Preparations of serum incubated for 15 or 30 min at 37°C, 18°C or 4°C were satisfactory. Those incubated for an hour or longer emitted increased nonspecific fluorescence, although the staining quality did not differ significantly from those incubated



Fig. 2. Indirect membrane immunofluorescence. The myofibrils remaining in conjunction with the muscle membrane were stained green at the cross-striations. $\times 400$.

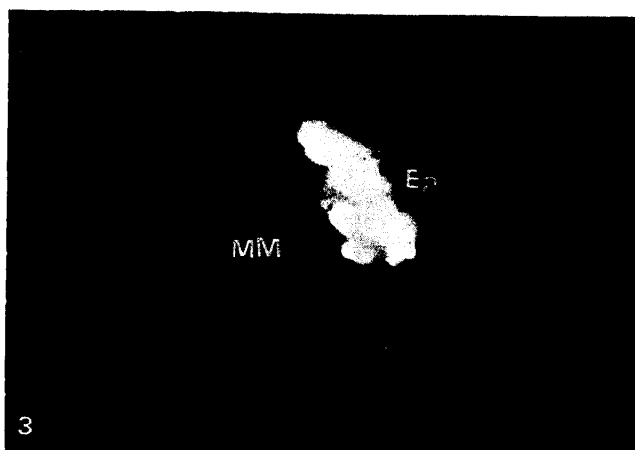


Fig. 3. Indirect membrane immunofluorescence with normal control serum. The muscle membrane (MM) was stained to a yellowish-brown hue and the motor endplate (Ep) to a yellowish tone. $\times 400$.

for 15 or 30 min. The experiment revealed that the incubation temperature was optimal at 4°C but adequate at 18°C. It was noted that washing with at least four changes of PBS was essential and that the specific fluorescence did not disappear even after washing with ten changes. When sera were diluted with PBS, the antibody titers of thymectomized patients were from 1 : 128 to 1 : 256 in contrast to nonthymectomized patient levels of 1 : 256 (type IIa) and 1 : 1024 (type IV). Hence, higher antibody titers were present in more advanced cases.

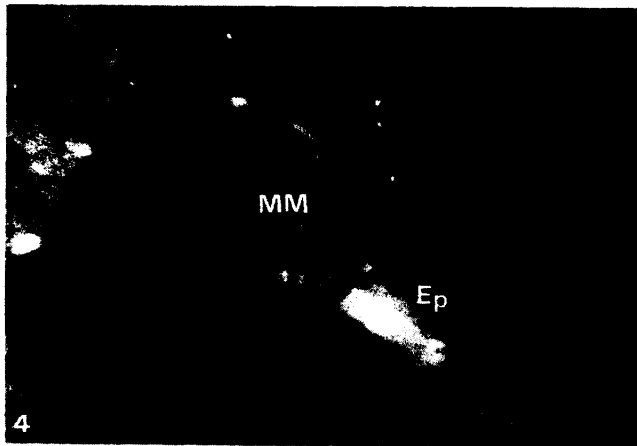


Fig. 4. Direct membrane immunofluorescence of a myasthenic patient. FITC-conjugated IgG stained the muscle membrane (MM) to a bright green. The motor endplate (Ep) was also stained deep green. $\times 400$.



Fig. 5. The double-stained preparation with FITC-conjugated myasthenic IgG and methylene blue. The blue-stained motor endplate (Ep) emitted the characteristic fluorescence. $\times 400$.

In direct staining with FITC-conjugated IgG of patients, the greenish tinge of the muscle membrane became allayed and a bright, green fluorescence resulted. The motor endplates were also stained a much deeper green (Fig. 4). With FITC-conjugated IgG of normal subjects, the muscle membrane was stained dark brown and the motor endplate yellow to yellowish-brown similar to the results of indirect membrane immunofluorescence. Brighter immunofluorescence in the antigen-antibody complex was more likely by incubating the mixture at 18°C or 4°C than at 37°C.



Fig. 6. Vital staining of prepared muscle membrane with methylene blue. Motor endplate (Ep) was stained blue. $\times 400$.

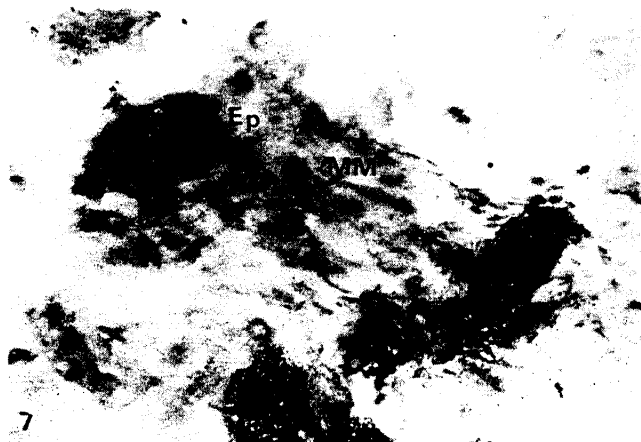


Fig. 7. Combined staining by hematoxylin-eosin and cholinesterase in prepared muscle membrane. The motor endplate (Ep) was stained yellowish-brown. $\times 400$.

In tests with FITC-conjugated patient IgG absorbed with muscle membrane, specific fluorescence by muscle membrane was not present, and the motor endplate was found to be stained yellowish-brown.

The double stained preparation with FITC-conjugated patient serum and methylene blue was examined using a dark field condenser for fluorescence microscopy and BV excitation to locate the motor endplates. The specimen was then re-examined with an OG filter for the light source instead of the BO filter. The blue-stained motor endplate emitted the characteristic fluorescence (Fig. 5). On slides of muscle membrane stained with methylene blue (Fig. 6) or with cholinesterase (Fig. 7), this green or yellow stained structure was readily identified as the motor endplate from its size, shape and distribution density.

DISCUSSION

Despite the strong presumption that MG is an autoimmune disease (8), a universal conclusion has not been reached that this condition is caused by immunological impairment, for the following reasons: (a) autoantibodies directed against striated muscles and against nuclei can be demonstrated only in a small proportion of cases; (b) intramuscular infiltrations of lymphocytes (lymphorrhages) are not necessarily extensive or marked; and (c) no specific antibody against the motor endplate has been demonstrated in spite of the essential abnormality and in spite of electrophysiologically impaired conduction of the motor nerve impulse at the neuromuscular junction. Our previous electron microscopic studies on MG patients using freeze-etched specimens provided evidence for structural irregularity of the transverse tubule orifices and demonstrated an increase in the diameter of particles on the surface of the skeletal muscle (9). This suggests the possible presence of immune complexes on the skeletal muscle surface. Our present findings as well as the microscopical photographs presented by Beutner *et al.* (10) and by Aarli and Closs (11) clearly demonstrate that these immune complexes result from anti-muscle membrane antibody which is present in the serum of MG patients. The presence of antibody activity against the motor endplates in such patients has been speculated since 1961 (12). In 1966 Namba and Grob (13) reported that specific immunofluorescence by structures resembling motor endplates was observed in 2 out of 3,000 MG sera. The ultimate reason for failure of demonstrating anti-motor endplate antibody in the past lies simply in the fact that frozen sections of muscle were fixed in ethanol or acetone. The membrane immunofluorescence technique (3) developed recently has made it practicable to verify the presence of IgG which in the past was demonstrated in plasma cells but not in lymphocytes. The present study represents an

attempt to apply the same principle used in examinations of preparations of fresh muscle membranes. Isolated muscle membranes tend to form aggregates and absorb dyes nonspecifically. In tests with IgG of normal subjects, the sites of muscle membranes and motor endplates were demonstrated by yellowish-brown fluorescence on BV excitation, as nonspecific reaction products. Rat muscle membranes and motor endplates were stained specifically by MG sera and emitted a bright green fluorescence that was clearly distinguishable from the fluorescence of healthy subject sera.

All MG patients reacted positively to anti-muscle membrane and anti-motor endplate antibodies. The antibody titers were higher in advanced cases than in earlier stages, and the titers had a tendency to decline following the removal of the thymus. The autoantibody against muscle membrane was found in patients with polymyositis as well as systemic lupus erythematosus. These patients, nevertheless, showed negative results to anti-motor endplate antibody.

Specific immunofluorescence by these antibodies has been confirmed in the present study. Specificity was more distinct by the direct test with FITC-conjugated IgG as compared to the serum sandwich technique, and specificity was absent in preparations with FITC-conjugated patient IgG absorbed with sufficient amounts of isolated muscle membrane.

It is important to determine whether the specific fluorescent positive structures on the muscle membrane were indeed motor endplates. The motor endplates of skeletal muscle stain blue (5) and yellowish-brown with cholinesterase (6). In the present study, motor endplates, identical in shape, size and distribution density to those seen under membrane immunofluorescence technique were confirmed on muscle membranes stained with methylene blue or cholinesterase. Furthermore, the blue-stained motor endplates emitted fluorescence in preparations stained directly with FITC-conjugated patient IgG, and subsequently stained with methylene blue and examined by fluoromicroscopy with BV excitation and OG primary filter.

The specific antibodies demonstrated against the muscle membrane and motor endplates of skeletal muscle may be involved in the developmental fluctuations, the diurnal variations and other variables operating to produce inconsistent therapeutic responses to anticholinesterase agents and thymectomy.

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