Specificity of muscle antibodies in sera from patients with myasthenia gravis and from immunized rabbits

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SYNOPSIS Citric acid extracts (CAE) of human skeletal muscle were prepared. Myasthenia gravis sera giving high titres in indirect haemagglutination test with this antigen, were selected. Rabbit antisera to CAE were prepared. The specificity of the two types of muscle antibodies was compared. Both patient sera and the rabbit antisera gave positive reactions in serological tests for muscle antibodies, but a difference in serological specificity was found.

Sera from some patients with myasthenia gravis (MG) contain antibodies to striated muscle. In immunofluorescence tests, different patterns of staining are described: cross-striational (Strauss *et al.*, 1960), sarcolemmal-subsarcolemmal (Aarli, 1972) and 'granular-mottled' (Cossio *et al.*, 1973). The different staining patterns may reflect different antigens in the muscle tissue.

Antibodies to skeletal muscle can also be produced by immunization of animals with muscle tissue homogenates or fractions thereof. The reactivity of these antibodies in immunofluorescence tests is similar to that observed with sera of patients with myasthenia gravis (Goldstein and Whittingham, 1967; Namba *et al.*, 1967).

Recent observations indicate that the muscle antibodies from immunized animals and from MG patients react with different antigens in skeletal muscle (Stroehmann *et al.*, 1973). The prime purpose of the present study was therefore to compare the specificity of the two types of muscle antibodies in reaction with a purified extract of human skeletal muscle tissue containing antigenic material.

METHODS

TISSUE PREPARATIONS Normal human skeletal muscle, usually from the thigh, was obtained at the Department of Surgery. Muscle tissue from rabbits was taken from the hind leg of healthy, non-immun-

ized animals. Citric acid extract (CAE) of human skeletal muscle was prepared as described by Espinosa and Kaplan (1968). The CAE was lyophilized and employed as earlier (Aarli, 1972).

SERA AND SERUM FRACTIONS MG sera with high titres in the indirect haemagglutination test for muscle antibodies (Aarli, 1972) were selected. Normal human sera were obtained from healthy blood donors. Normal rabbit sera were obtained from healthy, non-immunized animals. Incomplete anti-D serum was kindly provided by Dr K. Halvorsen of the Blood Group Laboratory.

Antiserum specific for human IgG globulin was prepared by immunization of a rabbit with the animal's own red cells agglutinated by human gamma-globulin solution purchased from AB Kabi Stockholm, Sweden (Larsen and Tønder, 1969).

Goat antiserum to rabbit serum was produced as described by Proom (1943). Rabbit antiserum to CAE was produced by intravenous immunization of a rabbit with 2 ml of a 50% suspension of the animal's own red cells, treated with tannic acid (1:60,000) and coated with CAE diluted 1:20. Five injections were given through three weeks and the first serum samples taken one week after the last injection.

Before use, all sera were heated at 56°C for 30 minutes (inactivation).

Fractionation of serum on Sephadex G-200 was performed according to Flodin and Killander (1962), using 0.05 M trisbuffer, pH 8.0, containing 0.146 M NaCl and 0.02% sodium azide. Samples of 2 ml serum were applied to a column (400 × 18 mm).

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Fractions of 5 ml were collected at a flow rate of approximately 20 ml/hr. The protein distribution with the effluents was registered at 254 nm in an Uvicord absorptiometer (LKB-produkter AB, Stockholm, Sweden). The fractions were absorbed with tanned red cells and used in the indirect haemagglutination test.

INDIRECT HAEMAGGLUTINATION TEST (IHA) The test with red cells sensitized with CAE was performed as described earlier (Aarli, 1972), except that rabbit red cells were employed. The reciprocal of the highest serum dilution giving agglutination was recorded as the titre.

ANTIGLOBULIN CONSUMPTION TEST (AGCT) The test was performed using human or rabbit muscle tissue sediments. For detection of antibodies in human serum, the procedure described earlier was followed (Aarli and Tønder, 1970). For detection of muscle antibodies in rabbit antiserum, the following modification of the same technique was applied: 10 mg of rabbit skeletal muscle tissue were used as antigen preparation. Goat antiserum to rabbit serum, diluted 1:32 was employed as antiserum. The titre of the goat antiserum against sensitized sheep red cells was 2048. A 1% suspension of sheep red cells sensitized with rabbit antiserum to sheep red cells, at a dilution two times higher than the agglutination titre, was used as indicator cells (SSB).

The consumption of antiglobulin serum recorded when using untreated tissue was designated *basal consumption*. When the tissue was treated with normal human serum, an increased consumption was obtained (*normal consumption*). With some MG sera, the consumption was four times or more greater than normal (*pathological consumption*). The consumption was recorded as the ratio between the titres before and after absorption.

IMMUNOFLUORESCENCE TESTS (IF) were performed mainly as earlier described (Aarli and Closs, 1972), but without Evans blue. For some experiments, paraffin-embedded ethanol-fixed sections were employed (Sainte-Marie, 1962). For inhibition experiments, the sections were incubated with serum for 20 minutes at 37°C and then washed in isotonic, phosphate-buffered saline, pH 7.2 (PBS) for 20 minutes with three changes of buffer.

RESULTS

DEMONSTRATION OF MUSCLE ANTIBODIES IN RABBIT ANTISERUM AND MG SERA

Indirect haemagglutination test (IHA) Rabbit antisera, normal (preimmune) serum, two MG

sera and normal human sera were titrated against tanned rabbit red cells coated with CAE. Antibodies to the CAE antigen were demonstrated in high titres both in rabbit antiserum and in MG sera (Table 1).

TABLE 1

INDIRECT HAEMAGGLUTINATION TEST USING RED CELLS SENSITIZED WITH CITRIC ACID EXTRACTS OF MUSCLE TISSUES. TITRES OBTAINED WITH HUMAN AND RABBIT SERA

Serum	Titre
Rabbit antiserum	32,000
Normal rabbit serum (pre-immune)	< 8
MG-12	128,000
MG-13	4,096
Normal human serum	< 8

Antiglobulin consumption test (AGCT) The AGCT was performed with rabbit antisera, normal (pre-immune) rabbit serum and PBS, and with serum MG 12, normal human serum and PBS, respectively. The results, as seen in Table 2, demonstrate the presence of antibodies to muscle tissue both in MG serum and rabbit antiserum.

TABLE 2

AGCT: TITRES OF ANTISERA AFTER ABSORPTION WITH MUSCLE TISSUE SENSITIZED WITH RABBIT ANTISERUM OR CAE/MG SERUM AND WITH NORMAL RABBIT/HUMAN SERA

Serum	Human muscle tissue Antiglobulin serum	Rabbit muscle tissue Goat anti-rabbit antiserum
Rabbit antiserum		64 (32)
Normal rabbit serum		256 (8)
PBS		1,024 (2)
MG 12	64 (128)	
MG 13	128 (64)	
Normal human serum	1,024 (8)	
PBS	4,096 (2)	

Antiglobulin consumption in parentheses, expressed as the ratio between titres before and after consumption.

Immunofluorescence (IF) tests IF patterns obtained with rabbit antisera were mainly of sarcolemmal/subsarcolemmal type. With transverse sections of the tissue, a distinct staining of the periphery of the fibres appeared (Fig. 1). In longitudinal sections, both sarcolemmal/subsarcolemmal staining and a variable degree of staining of cross-striations were demonstrated (Fig. 2). The same patterns were obtained with labelled F(ab')₂—of IgG MG 12. Although small

FIG. 1. *Ethanol-fixed rabbit skeletal muscle incubated with FITC-labelled rabbit antiserum to CAE, diluted 1:16. Sarcolemmal fluorescence. Film: Ilford HP4, exposure time 40 s.*

FIG. 2. *Acetone-fixed rabbit skeletal muscle incubated with FITC-labelled rabbit antiserum to CAE. Cross-striational and sarcolemmal fluorescence. Film: Ilford HP4, exposure time 10 s.*

differences could be seen in areas of the sections, these were of a quantitative nature, and the localization of the specific fluorescence was indistinguishable with the two sera.

NATURE OF ANTIBODIES In order to study which Ig class of the human and rabbit sera was responsible for the antibody activity against muscle tissue, serum MG 13 and rabbit antiserum were subjected to gel filtration on Sephadex G-200. The haemagglutination titres of the fractions were recorded. With the rabbit antiserum, fractions both from the first and the second protein peak agglutinated the cells. This indicates the presence of muscle antibodies both in the IgM and the IgG fractions. With MG serum, however, no agglutination occurred with the fractions from the ascending part of the first peak, while the fractions from the second peak agglutinated the cells, indicating mainly

muscle antibodies of IgG type in this serum (Fig. 3).

INHIBITION EXPERIMENTS Experiments were performed in order to study whether the muscle antibodies of the two sera are directed against the same antigenic determinant(s).

The antiglobulin consumption test was performed with normal human serum and serum MG 12. Before incubation with the tissue, the sediments were incubated for 30 minutes at 37°C with 2 ml rabbit antiserum. Pre-treatment of the tissue with rabbit antiserum did not reduce the binding of human antibodies to the tissue. Corresponding experiments were performed with rabbit antiserum and muscle tissue pre-treated with serum MG 12. The MG serum did not interfere with the binding of rabbit antibodies to the tissue (Table 3).

Blocking experiments were also performed with the direct IF test. Sections of acetone-fixed

FIG. 3. Titres in indirect haemagglutination test with fractions obtained by gel filtration of rabbit antiserum to CAE (above) and serum MG 13 (below) on a Sephadex G-200 column. — Per cent transmission in effluents; ● titres of fractions.

TABLE 3

AGCT: EFFECT OF PRETREATMENT OF TISSUE WITH NORMAL SERUM, RABBIT ANTISERUM TO CAE AND MG SERUM

Pretreatment	Incubation with	Titre of antiserum
PBS	Normal human serum	1,024 (8)
Rabbit antiserum	Normal human serum	1,024 (8)
PBS	MG 12	64 (128)
Rabbit antiserum	MG 12	64 (128)
PBS	Normal rabbit serum	256 (8)
MG 12	Normal rabbit serum	256 (8)
PBS	Rabbit antiserum	64 (32)
MG 12	Rabbit antiserum	64 (32)
Rabbit antiserum	MG 12	64 (32)

Antiglobulin consumption in parentheses, expressed as the ratio between titres before and after consumption.

rabbit skeletal muscle were incubated with PBS, normal human serum, rabbit antiserum, and serum MG 12, respectively. The sections were then washed three times in PBS and incubated with labelled MG 12 and rabbit antiserum, in different experiments. The rabbit antiserum did not block the reaction with the MG serum and *vice versa* (Table 4).

TABLE 4

IF TEST FOR MUSCLE ANTIBODIES: BLOCKING EXPERIMENTS

Blocking with	Immunofluorescence with	
	Rabbit antiserum	MG 12
PBS	+	+
Normal rabbit serum	+	—
Normal human serum	—	+
Rabbit antiserum	—	+
MG 12	+	—

DISCUSSION

Sera of some patients with myasthenia gravis contain antibodies to antigens present in acid extracts of skeletal muscle. Immunization of rabbits with such extracts results in production of muscle antibodies. The patient sera and the antisera both gave positive reactions in AGCT, IHA, and IF tests for muscle antibodies.

The antibody activity of the myasthenia gravis serum is mainly of IgG type. This is probably the result of a chronic antigenic stimulation, while the IgM antibody activity found in experimental antisera reflects the acute response of an active immunization.

The results of the inhibition experiments show that the rabbit antiserum does not block the binding of antibodies from myasthenia gravis sera to muscle tissue in the AGCT. Neither does pretreatment of the tissue with patient serum abolish the reaction with rabbit antiserum. In IF tests, pretreatment with rabbit antiserum did not interfere with the staining produced by labelled patient serum, as pretreatment with unlabelled patient serum did. On the basis of these experiments, it was concluded that the antibodies of myasthenia gravis sera react with different antigenic determinants than the experimentally produced antibodies.

Espinosa and Kaplan (1970) have demonstrated several antigens in CA extracts of heart muscle, partly cross-reacting also with skeletal muscle. The present data do not allow identification of the antigenic activity to a single component of the extracts. They are, however, comparable with those reported by Stroehmann *et al.* (1973), who found antibodies to 'the myosin complex of skeletal muscle' both in myasthenia gravis patients and in rabbits immunized with myosin-actomyosin-fractions. The IF patterns obtained were identical, with cross-striations as the dominating feature. Double diffusion tests revealed, however, that the antibodies were directed against different antigens of skeletal muscle. The CAE antigen gave very weak precipitation lines (Aarli, 1972), but other serological tests revealed, however, a difference in the antibody specificity with the two serum types.

None of the animals used for immunization developed clinical signs of muscular disease. Similar findings are reported by other authors (for references, see Kaufman *et al.*, 1969). The experimentally produced antibodies therefore seem to be of no pathogenetic significance.

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