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Received September 7, 1960. P.S.E.B.M., 1960, v105.

Immunofluorescence Demonstration of a Muscle Binding, Complement-Fixing Serum Globulin Fraction in Myasthenia Gravis.* (26051)

ARTHUR J. L. STRAUSS,† BEATRICE C. SEEGAL, KONRAD C. HSU, PETER M. BURKHOLDER, WILLIAM L. NASTUK AND KERMIT E. OSSERMAN

Depts. of Microbiology and Physiology, College of Physicians and Surgeons, Columbia University; Dept of Medicine, Myasthenia Gravis Clinic, Mount Sinai Hospital, New York; Dept. of Pathology, New York Hospital-Cornell Medical Center

Serum from some patients with myasthenia gravis has been shown to have a heat-labile cytolytic effect on sartorius muscle of the frog (1). Serum complement levels in many myasthenic patients in various stages of the disease often vary greatly beyond both the upper and lower limits of normal(2,3). These findings led to the speculation that some complement fixing immunologic system might be implicated in the pathogenesis of myasthenia gravis. In a test of this hypothesis, the immunofluorescence technic of Coons and Kaplan(4) was employed, using serum and skeletal muscle from patients with the disease.

We have demonstrated that the globulin fraction, prepared by 20% sodium sulfate precipitation of serums pooled from 10 myasthenic patients in the early progressive phase of their illness, as distinct from the normal serum globulin fraction prepared in like manner, has the following characteristics: 1. Ability to localize regularly and consistently in alternate striations of human skeletal muscle, both normal and myasthenic, as well as in skeletal muscle of one other mammalian species. This property was made evident by direct tagging of both myasthenic and normal globulins with fluorescein isothiocyanate. 2. Ability, when untagged, to block adherence of fluorescein tagged myasthenic globulin to skeletal muscle. 3. Ability, when once localized in skeletal muscle, to bind whole guinea pig complement. This fixation, *in vitro,* of heterologous complement was demonstrated by use of fluorescein conjugated rabbit antibodies to guinea pig complement. We have also shown that some individual whole myasthenic serums, including some of those utilized in the preparation of the myasthenic globulin pool, as distinct from normal human serums, are (1) capable of blocking adherence of fluorescein tagged myasthenic globulin to skeletal muscle striations, and (2) seemingly capable of fixing guinea pig complement in much the same way as does the myasthenic globulin fraction.

Materials and methods. Source of serums, and preparation of globulin pools. Serums from patients with myasthenia gravis were obtained on the wards and in the outpatient departments of Mount Sinai Hospital, and Columbia-Presbyterian Medical Center, New York. Diagnosis in each case had been established by history, physical findings, symptoms, and positive response to one or more anticholinesterase drugs. Control serums were taken from 12 medical house officers with an age and sex distribution comparable to that of the myasthenic patients. All were essentially healthy and denied histories of major system disease.

* Aided by grants from Nat. Inst. of Health and Helen Hay Whitney Foundation.

† Post-Doctoral Research Fellow, Nat. Inst. of Health.

All blood samples were collected with sterile precautions and the serums stored without preservative at 4°C, from 7 days to 6 months before use. Our experience has been that these storage conditions favor preservation of serum globulins suitable for tagging with fluorescein isothiocyanate. When many of these serums were drawn, we did not anticipate their use in a study involving serum complement activity; therefore no attempt was made to store aliquots in the deep freeze to preserve complement titers.

Ten serums were selected from among 105 collected from 96 myasthenic patients. The following criteria were used for selection of serum for this pool: (1) Patients shall have had clinical manifestations of myasthenia gravis for less than 3 years, preferably for less than one year, (2) generalized disease involving bulbar and peripheral musculature shall be manifest, (3) the disease should be progressive or acutely exacerbating at time of blood collection, (4) patients shall have some evidence of thymic pathology by radiography, or as assessed by observation at time of thymectomy. The patients shall not have undergone thymectomy prior to sampling; information regarding thymic pathology seen at surgery would, therefore, be used retrospectively. Seven serums came from patients with a history of clinical disease of no more than one year; the remaining serums from patients with less than a 3 year history of clinically apparent myasthenia gravis. Accordingly, serums from 10 patients were pooled in the following proportions to make a serum pool of 25 cc.

Patient	Age	Clinical course at time of blood collection	Amt of serum, cc
1. ♀	30	3 mo history of oculobulbar and peripheral weakness, positive response to anticholinesterase medication. Thymectomy, 10 days post sampling revealed hyperplastic thymus.	7
2. ♀	53	8 mo history of oculobulbar and peripheral weakness. Positive response to anticholinesterase medication. Thymectomy, one day following sampling, revealed benign thymoma.	2
3. ♂	14	3 yr history of progressive peripheral and oculobulbar weakness. Positive response to anticholinesterase medication. Thymectomy next day revealed thymic hyperplasia.	2
4. ♀	15	1½ yr history of progressive generalized disease. Positive response to anticholinesterase medication. Thymectomy, 5 days post sampling, revealed hyperplastic thymus.	2
5. ♂	26	10 mo history of generalized and progressive weakness. Positive response to anticholinesterase medication. Thymectomy 5 days post sampling revealed benign thymoma.	2
6. ♂	53	2 yr history of generalized disease. Positive response to anticholinesterase medication. One yr prior to sampling, patient underwent exploratory thoracotomy, at another hospital; inoperable thymoma found.	2
7. ♀	32	3 yr history of oculobulbar and generalized disease. Positive response to anticholinesterase medication. Thymectomy, 2 wk post sampling, revealed thymic hyperplasia.	2
8. ♂	48	One yr history of generalized & oculobulbar symptoms. Positive response to anticholinesterase medication. Subsequent thymectomy revealed malignant thymoma.	2
9. ♀	55	One yr history of generalized & oculobulbar symptoms. Positive response to anticholinesterase medication. Subsequent thymectomy revealed thymoma.	2
10. ♀	48	Approximately 6-8 mo history of generalized progressive disease. Evidences of enlarged thymic shadow by x-ray.	2

Aliquots from 10 myasthenic serums were combined as given above to make a 25 cc serum pool; a similar pool was prepared from 12 serums from healthy individuals. Each pool was absorbed with 6 ml of packed sterile human erythrocytes, AB positive, washed 3 times with 0.85% NaCl.

Preparation of globulins from myasthenic and normal serum pools. The serum pools were diluted with equal volumes of sterile 0.85% NaCl and to each 10 ml of serum saline mixture, 2.04 g of anhydrous sodium sul-

fate were added. After standing at room temperature for 2 hours or more, the suspensions were centrifuged and precipitates dissolved in volumes of distilled water equal to two-thirds of initial volumes of diluted serum pools. The globulins were reprecipitated by adding 1.93 g of sodium sulfate for each 10 ml of distilled water added. The suspensions were again centrifuged and a third precipitation carried out in the same manner. Final precipitates were dissolved in minimum amounts of distilled water, dialyzed against running cold tap water until first appearance of reprecipitation, then dialysis was continued against 0.9% NaCl with mechanical stirring overnight at 4°-6°C. The resulting globulin solutions were adjusted to a concentration of 20 mg/ml with normal saline, passed through Seitz Filters, and stored in the refrigerator at 4°-6°C without preservative. Paper electrophoresis of myasthenic and normal serum globulin fractions thus prepared revealed albumen free preparations consisting of alpha, beta, and gamma globulins. Curves for myasthenic and normal pools were completely superimposable. Immunoelectrophoresis of both globulin preparations against horse anti-human whole serum revealed essentially identical results for myasthenic and normal globulin pools.

Portions of both myasthenic and normal globulins were conjugated with fluorescein isothiocyanate‡ according to the methods of Riggs(5) and Marshall *et al.*(6). Fluorescein conjugated globulins were dialyzed with sterile precautions against 0.01 molar phosphate buffered saline (pH 7.2) at 4°-6°C for 6 days to eliminate free fluorescein isothiocyanate. Conjugates were subsequently absorbed with rat and mouse liver powders to remove non-specific fluorescence.

Guinea pig complement. Hyland§ Dried Complement (guinea pig) prepared from healthy male or non-pregnant female guinea pigs was used (Lot #370M26) throughout these experiments. The equivalent of 7 cc of dried guinea pig complement was reconstituted with 5 cc of diluent supplied by Hyland Laboratories. All samples so prepared were assayed by the Serology Laboratories of Presbyterain Hospital, New York, and found to have hemolytic complement activities of 1:42 by dilution. Dilutions used for our studies ranged from 1:15-1:30. A calcium-magnesium saline solution (instructions for preparation accompany packaged dried complement) was used for all complement dilutions.

Fluorescein conjugated rabbit anti-guinea pig complement. This conjugated anti-serum was prepared according to technics described elsewhere(7). Antigen used to prepare this anti-serum in rabbits was sensitized sheep red cell stromata that had been exposed to guinea pig complement. The resulting rabbit anti-serum globulin was absorbed with sheep red cell stromata, and sensitized sheep red cell stromata, free of fixed guinea pig complement, and the resulting anti-guinea pig complement was tagged with fluorescein isothiocyanate.

Collection and preparation of tissues, staining with fluorescent globulin conjugates: The following tissues were utilized in this study:

Human skeletal muscle biopsies.

A. Intercostal muscle biopsy taken at thymectomy from patient #1, a myasthenic who contributed to the serum pool.

B. Forearm muscle biopsy from a 71-year-old female myasthenic.

C. Rectus Abdominis muscle biopsy from a 43-year-old myasthenic undergoing hysterectomy.

D. Forearm muscle biopsy from a 33-year-old female patient with myotonic dystrophy.

E. Pectoralis muscle biopsy from a six-year-old male undergoing open heart surgery for a congenital cardiac defect.

F. Rectus abdominis muscle biopsy from a 43-year-old male undergoing subtotal gastrectomy for peptic ulcer.

Rat skeletal muscle

G. Abdominal skeletal muscle biopsy from a healthy male Sprague-Dawley rat.

Human cardiac muscle

H. Auricular appendage containing myocardium, from patient with rheumatic valvular disease who had undergone mitral commissurotomy.

Human myometrium (smooth muscle)

‡ Sylvana Chemical Co., Orange, N. J.

§ Hyland Labs., Los Angeles, Calif.

I. and J. Two biopsies taken from uteri at hysterectomies from 2 myasthenic women. One of these 2 patients also provided skeletal muscle biopsy C. described above.

Thymic tissue: from 2 myasthenic patients.

K. Thymic hyperplasia from patient #1, who had contributed serum to pool and intercostal muscle biopsy A., described above.

L. Thymoma tissue from patient #9 of myasthenic serum pool.

All tissues were taken at time of surgical removal and quick frozen in sealed Pyrex test tubes surrounded by a slush of dry ice and butanol, then stored in a dry ice freezer at −65°C until use. All tissues were sectioned on an A-O Rotary Microtome at a setting of 1-2 μ in the longitudinal plane in a Harris Cryostat at −20°C. Sections were mounted on 1 mm thick slides as required for dark field observation with an ultraviolet source. Before 'staining' with fluorescein conjugated globulins, sections were fixed in 95% Ethanol for 30 seconds. Alcohol was washed from the slides with 0.01 M phosphate buffered saline, pH 7.2. Slides were then rinsed in buffered saline for 10 minutes, then wiped as dry as possible without removing fluid overlying sections. Slides were then transferred to moist chambers and submitted to the respective control and test staining procedures to be described. Following completion of staining schedules, sections were again rinsed in buffered saline for 10 minutes, mounted in buffered glycerin (pH 7.2), and covered with No. 1 cover slips. All sections were examined by means of an ultraviolet light source using a 200 Watt high pressure mercury vapor arc lamp of 2500 stuble mounted in a Reichert Fluorex Unit. Five filters were used for visualization, 2 red excluding, 1 ultraviolet transmitting, 1 BG 12, and 1 ultraviolet excluding filter over the ocular.

Photography. A Leica Camera Body was used with a Reichert KAM attachment to the microscope. All photographs were taken on AGFA ISOPAN RECORD film, at ASA 4000 under 10 x 43 magnification with an average exposure of 15 minutes.

Results. Sections of skeletal muscle from all human biopsies, normal, myasthenic and dystrophic were treated with the fluorescein conjugated myasthenic globulin fraction, undiluted, or diluted 1:2.5, 1:4, and 1:5, two drops per section, for 30 minutes, and examined following a 10 minute rinse with 0.01 M phosphate buffered saline, pH 7.2. In every section, brilliant apple green fluorescence was observed in alternate striations consistently throughout every longitudinally sectioned fiber (Fig. 1). A more or less punctate fluorescence was seen in fibers cut in transverse plane. In some sections, especially where the fluorescein tagged myasthenic globulin was applied undiluted, there was a variable amount of fluorescence in the sarcolemma. There was no fluorescence of this color value or intensity in the connective tissue stroma or blood vessels included in these sections. Sections stained with fluorescein conjugated normal human globulin fraction *did not* fluoresce in any locus (Fig. 2). Rat skeletal muscle, when treated similarly with fluorescein conjugated myasthenic globulin, evidenced focal localization of fluorescent material in alternate striations. Normal human fluorescein tagged globulin produced no fluorescence in rat muscle. Sections of auricular appendage myocardium failed to evidence fluorescence when treated with either myasthenic or normal fluorescein conjugated globulin fractions, and sections of myometrium were also negative. Sections of thymic tissue from 2 myasthenic patients likewise were not stained by fluorescein conjugated myasthenic and normal globulin fractions.

Several sections of skeletal muscle from each biopsy were treated with myasthenic globulin unconjugated and undiluted, for 15 minutes prior to treatment with fluorescein tagged myasthenic globulin, to determine that dilution of fluorescein tagged myasthenic globulin which would still be capable of staining sections in the face of a competitive block of binding sites with untagged globulin. Whereas a 1:5 dilution of fluorescein conjugated myasthenic globulin produced bright fluorescence in muscle sections *not* previously treated with untagged, undiluted myasthenic globulin, use of this dilution on sections previously treated with unconjugated, undiluted myasthenic globulin, produced little or no fluorescence. Dilutions 1:4 and 1:2.5 of the

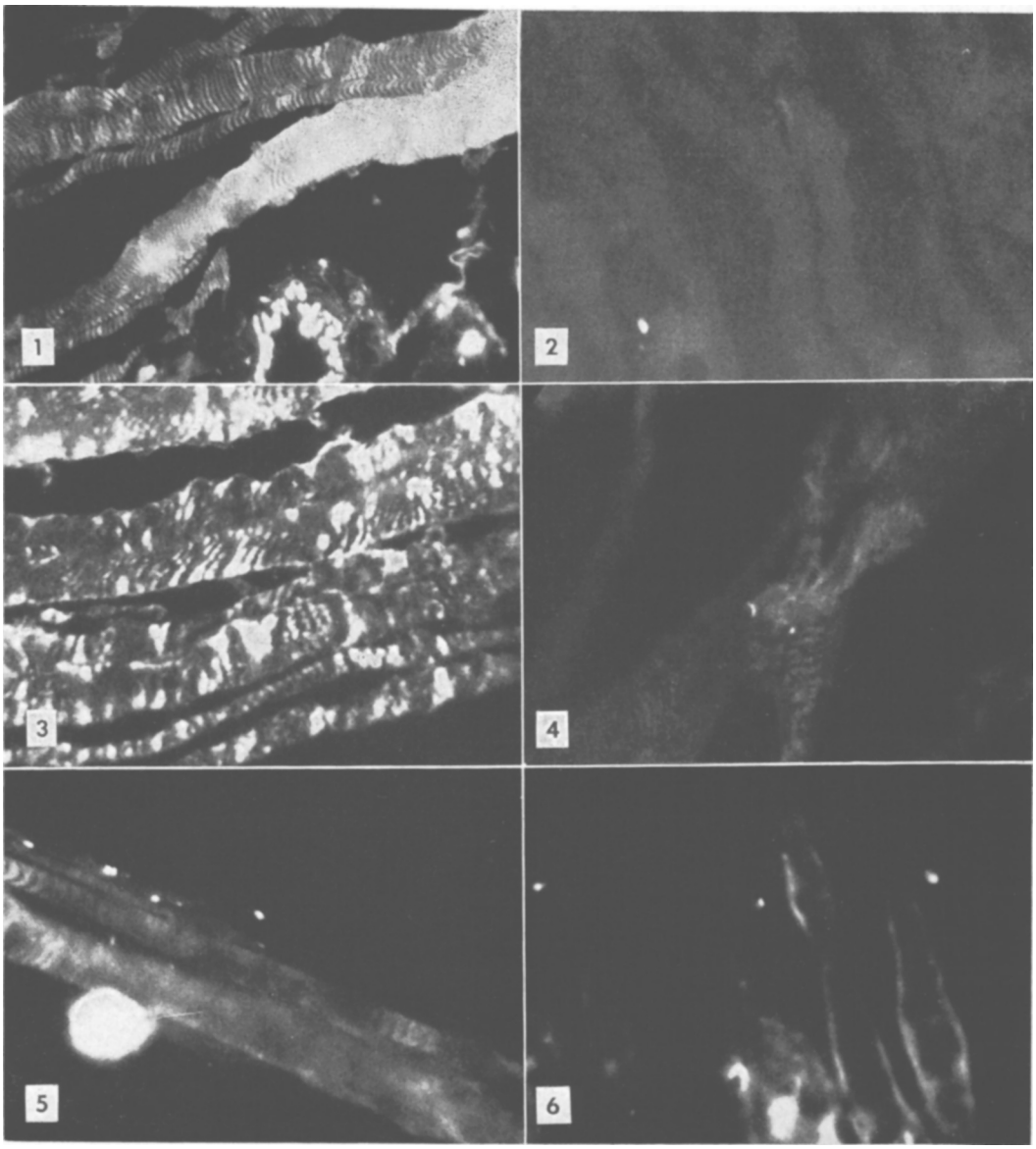

FIG. 1. Section of myasthenic skeletal muscle biopsy A. stained with fluorescein labelled globulin prepared from a pool of 10 myasthenic serums. Note delicate fluorescent striations. Haziness of fluorescence in mid-diagonal fiber, due to the fact that it is in part out of focus. Wavy fluorescence in lower middle field represents autofluorescence in tunica elastica of an arteriole. Magnification 350 ×.

FIG. 2. Section of myasthenic skeletal muscle biopsy B. treated with fluorescein labelled normal human globulin. No fluorescence. Magnification 350 ×.

FIG. 3. Section of myasthenic skeletal muscle biopsy A. treated successively with untagged, undiluted myasthenic globulin, guinea pig complement, and fluorescein labelled rabbit anti-guinea pig complement. Note disruption of fluorescent striations. Magnification 350 ×.

FIG. 4. Section of myasthenic skeletal muscle biopsy A. treated successively with undiluted untagged myasthenic globulin, heat inactivated guinea pig complement, and fluorescein tagged rabbit anti-guinea pig complement. Note that fluorescence, though present, is markedly reduced when compared with section in Fig. 3. Magnification 350 ×.

FIG. 5. Section of myasthenic muscle B. treated with serum of a patient with paroxysmal myoglobinuria, then successively with guinea pig complement and fluorescein tagged rabbit-anti-guinea pig complement. Large fluorescent spot in lower left field represents bubble in

myasthenic globulin, fluorescein tagged showed very appreciable diminution of fluorescence in striations when sections had been previously treated with untagged, undiluted myasthenic globulin. It was concluded, therefore, that untagged myasthenic globulin could competitively inhibit the adherence of fluorescein conjugated myasthenic globulin fraction to skeletal muscle striations. An interesting, as yet unexplained observation was that when skeletal muscle sections, were successively treated with untagged, undiluted normal globulin (15 minutes) and then with fluorescein tagged myasthenic globulin, undiluted or in dilutions to 1:5, fluorescent striations appeared somewhat crisper to the eye than when fluorescein tagged myasthenic globulin was used alone.

A limited series of experiments has been completed in an effort to determine the effect of individual myasthenic serums in blocking the staining produced by the fluorescein conjugated myasthenic globulin fraction. Included in this study were 10 individual myasthenic serums which also contributed to the myasthenic serum pool, and 3 additional myasthenic serums taken from patients with disease of less than one year's duration. No effort has been made rigidly to quantitate these observations, but an acceptable generalization can be made that all muscle sections treated with these selected myasthenic serums for from 15 to 45 minutes evidenced appreciable diminution in intensity of fluorescent staining when sections were treated subsequently with fluorescein conjugated myasthenic globulin fraction 1:4 for 25 minutes; compared with sections treated with fluorescein tagged myasthenic globulin 1:4, alone. This generalization assumes more significance in view of the fact that prior treatment of skeletal muscle sections with 6 normal serums resulted in a *sharper* delineation of fluorescent striations produced by subsequent treatment of sections with fluorescein conjugated myasthenic globulin, 1:4.

In view of the apparent demonstration of a muscle binding globulin exclusive to the myasthenic globulin pool, and the observations of Nastuk, *et al.*, with respect to alterations in serum complement activity in myasthenia gravis(2,3), a test was made of the ability of the untagged myasthenic globulin fraction, when bound to skeletal muscle, to fix guinea pig complement. The technic of Klein and Burkholder(8) for demonstration of complement fixation by fluorescence microscopy was used. Sections of human skeletal muscle were treated successively with: 1. untagged, undiluted myasthenic globulin, 2. guinea pig complement in appropriate dilution, and 3. fluorescein conjugated rabbit anti-guinea pig complement. Results, together with control observations, are presented in Table I. Experiments were carried out in identical fashion with sections from 2 myasthenic muscle biopsies A. and B., and one normal muscle biopsy, F. The localization of guinea pig complement by this immunofluorescence technic is similar to that of the fluorescein conjugated myasthenic globulin, but there are obvious differences. Whereas the fluorescein conjugated myasthenic globulin alone localizes in striations in a rather discrete manner, complement localization, in sections treated with untagged myasthenic globulin, as demonstrated by the use of fluorescein conjugated rabbit anti-guinea pig complement, results in a fluorescent pattern of parts or particles of alternate skeletal muscle striations. Striations and sarcolemma in the latter sections also seem disrupted. On inspection with ultraviolet dark field illumination, *none* of the control sections (Table I) treated with guinea pig complement evidenced marked disruption of muscle fiber membranes. It is conceivable that this disruption might be due to cytolytic activity of complement *fixed* by the myasthenic globulin fraction. This awaits further study.

Finally, we screened 31 myasthenic serums from 30 individual patients, 11 individual normal serums, and 5 serums from other generalized myopathies; these were tested for their

glycerin. Note minimal fluorescence in some striations. Magnification 350 ×.

FIG. 6. Section of normal human skeletal muscle biopsy F treated with serum from a patient with acute dermatomyositis, and successively with guinea pig complement and rabbit anti-guinea pig complement, fluorescein tagged. Note sarcolemmal fluorescence. Magnification 350×.

TABLE I. Histoserological Demonstration of Fixation of Guinea Pig Complement to Sections of Human Skeletal Muscle Previously Treated with Serum Globulin from Patients with Myasthenia Gravis.

Successive treatment of muscle sections with:						
1.	Wash, min.	2.	Wash, min.	3.	Wash, min.	Fluorescence
Buffer (15 min.)	30	Buffer (1 hr)	30	Rab. anti-G.P. comp. "F" (1 hr)	10	None
Idem	30	G. P. comp. 1:30 (1 hr)	30	*Idem*	10	"
Myasthenic glob. untagged, undiluted (15 min)	30	*Idem*	30	"	10	Alternating fluorescent striations (Fig. 3)
Idem	30	Heat inactivated (56°C, 30 min.) G. P. comp. 1:30 (1 hr)	30	"	10	None in most sections. Few faint striations in others (Fig. 4)
"	30	Rab. anti-G.P. comp. "F" (1 hr)	10	—	—	None
Normal glob. untagged, undiluted (15 min.)	30	G. P. comp. 1:30 (1 hr)	30	Rab. anti-G.P. comp. "F" (1 hr)	10	"
Idem	30	Heat inactivated (56°C, 30 min.) G. P. comp. 1:30 (1 hr)	30	*Idem*	10	"
"	30	Rab. anti-G.P. comp. "F" (1 hr)	10	—	—	"

ability to fix complement in relation to skeletal muscle. Successive treatment of muscle with the whole serum in question, guinea pig complement, and fluorescein conjugated rabbit anti-guinea pig complement was carried out, and the following observed: 1. Sections treated with serums from 8 of 10 myasthenics included in the original myasthenic serum pool, when successively treated with guinea pig complement and fluorescein tagged rabbit anti-guinea pig complement evidenced fluorescent striations. Skeletal muscle sections exposed to 2 remaining serums included in the original pool failed to fix guinea pig complement; hence, no fluorescence was seen. 2. Sections treated in like manner with serums from all of 5 other myasthenics with disease of less than one year's duration, when treated successively with guinea pig complement and fluorescein tagged rabbit anti-guinea pig complement evidenced fluorescent striations. 3. Not all myasthenic serums gave rise to fluorescent skeletal muscle sections by this technic; 16 serums taken from individual patients without regard to clinical course gave negative or equivocal results. (Subsequent check revealed that 5 of the 16 serums had come from patients with clinically apparent myasthenia gravis of less than 2 years' duration). 4. One serum sample drawn from patient #1 of the myasthenic serum pool, some 4 months post-thymectomy, fixed complement by this technic. 5. *No sections* treated with the *11 normal serums*, then successively with guinea pig complement and flourescein conjugated rabbit anti-guinea pig complement, evidenced fluorescence in striations. 6. One serum from a patient with exacerbating paroxysmal myoglobinuria gave rise to fluorescent striations in a skeletal muscle section following use of guinea pig complement, and fluorescein conjugated rabbit anti-guinea pig complement (Fig. 5). 7. A section of normal human skeletal muscle treated with serum from a patient with acute dermatomyositis, and successively treated with guinea pig complement and fluorescein tagged rabbit anti-guinea pig complement evidenced sarcolemmal fluorescence (Fig. 6). 8. Single serum samples from 3 patients with progressive muscular dystrophy, idiopathic acute polymyositis, and myopathy associated with periarteritis nodosa, respectively, yielded negative results by this technic.

Discussion. These preliminary observations are provocative of several questions which await further study. Is this muscle binding, complement-fixing globulin factor peculiar to one or is it a property of several of the serums included in the myasthenic pool? Our data with respect to blocking and complement fixation with individual myasthenic serums on skeletal muscle suggest its presence in 8 of 10 serums comprising the pool. Is what we have demonstrated by fluorescence microscopy a true immunologic phenomenon? Is it a reaction between a specific muscle antigen and an auto-antibody; if this should be the case, what is the nature of the antigen involved? The ability of this system to fix complement suggests an immune phenomenon. The more classical technics, agar diffusion, precipitation methods, agglutination, and test tube complement fixation should be employed to further this study. Does this material, unique to the myasthenic globulin fraction, represent gamma globulin, or possibly alpha or beta globulin? Our globulin preparations contain alpha, beta, and gamma fractions. Further separation of myasthenic pooled globulins into alpha, beta, and gamma components is under way.

Other questions include the origin of the factor peculiar to the myasthenic globulin and its relation, if any, to the pathogenesis of the disease. Is its presence in any way related to the thymic pathology seen in association with myasthenia gravis? What relation do these observations bear to the derangement in serum complement in myasthenia gravis? Is the presence of a complement-fixing, muscle binding globulin in serum a feature of myasthenia gravis exclusively, or can it be seen in other myopathies?

Summary. 1. A muscle binding, complement fixing component has been demonstrated in the crude globulin fraction of a pool of serum from 10 patients with myasthenia gravis of recent onset and progressive character, by means of the immunofluorescence technic. This component could not be demonstrated in a similarly prepared normal serum globulin pool. Untagged myasthenic globulin was shown to block competitively adherence of fluorescein tagged myasthenic globulin to skeletal muscle striations; whereas prior treatment of muscle sections with normal serum globulin intensified staining with fluorescein tagged myasthenic globulin. Individual myasthenia gravis serums included in the pool also blocked staining with fluorescein conjugated myasthenic globulin. Normal serums did not block adherence of the fluorescein tagged myasthenic globulin to skeletal muscle. 2. The myasthenia gravis globulin fraction was shown to fix guinea pig complement to human skeletal muscle, by successive treatment of muscle sections with myasthenic globulin, guinea pig complement, and fluorescein conjugated rabbit anti-guinea pig complement. Normal serum globulin failed to fix complement by this technic. 3. Thirty-one myasthenia gravis serums were screened, with 11 normal serums, and 5 serums from patients with other generalized myopathies, for their ability to fix guinea pig complement to skeletal muscle. Thirteen myasthenic serums gave unequivocal evidences of complement fixation, using the immunofluorescence technic. No normal serums fixed complement. Serum from one patient with paroxysmal myoglobinuria also fixed complement. Serum from one patient with acute dermatomyositis gave rise to fluorescence in the sarcolemma when layered onto skeletal muscle followed successively by guinea pig complement and fluorescein conjugated rabbit anti-guinea pig complement.

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